Investigation of the Role of the Nucleus Accumbens in Amphetamine-Induced 50 kHz Ultrasonic Vocalizations in the rat

by

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Abstract

There is extensive evidence that the mesolimbic dopamine system underlies the production of 50 kHz ultrasonic vocalizations in rats. In particular, the shell of the nucleus accumbens is associated with generation of frequency modulated 50 kHz calls (a specific type of 50 kHz call which can be subdivided into various subtypes). There is also evidence that amphetamine administered systemically preferentially increases the proportion of trill and step calls compared to other frequency modulated 50 kHz subtypes. The purpose of this study was to investigate the effect of drug administration route and the role of the nucleus accumbens shell in amphetamine-induced 50 kHz call profile in the rat. Three experiments investigated this by using subcutaneous and intra-accumbens microinjections of amphetamine, as well as procaine (a local anesthetic) blockade of the nucleus accumbens. Ultrasonic vocalizations were recorded digitally from 24 rats and were analysed for sonographic structure based on general call parameters. The results of the three experiments were partially supportive of the hypotheses. Systemic amphetamine was found to induce greater bandwidth in 50 kHz calling compared to spontaneous calls in a vehicle condition. Systemic amphetamine was also found to preferentially increase the proportion of trill and step subtypes compared to vehicle. Moreover, there was no difference in the proportions of 50 kHz subtypes resulting from intracerebral or systemic application of amphetamine. There was, however, a significant difference for bandwidth, with systemic amphetamine inducing greater bandwidth over intraaccumbens application. Procaine blockade of the nucleus accumbens shell paired with subcutaneous amphetamine produced no difference in bandwidth of calls compared with those after a vehicle pre-treatment similarly paired. There was no reduction in the proportions of trill and step 50 kHz subtypes as well, with the procaine condition showing significantly greater proportion of step calls. The results of the study support a role for the

nucleus accumbens shell in the amphetamine-induced changes on 50 kHz call profile. They also indicate there are more regions and pathways involved in generating 50 kHz calls than the projections from the ventral tegmental area to the nucleus accumbens. The implications of this work are that frequency modulated 50 kHz subtypes may be generated by distinct neurophysiological mechanisms and may represent a profitable avenue for investigating different circuits of 50 kHz call categories in the rat.
Keywords: Accumbens, Ultrasonic-vocalizations, pharmacology, amphetamine, 50-kHz.

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Communication in Animals

Communication in animals includes processes of both signal production as well as that of reception. This communicative process requires not only the conveyance of a signal (coded and transmitted in some fashion) but the potential induction of behavioural response from the target organism (Brudzynski, 2005). However, this dyadic perspective (one signaler to one receiver) does not provide a complete understanding, as communication networks can be established among many individuals (McGregor, 2005). Nevertheless, both perspectives are grounded on the fundamental characteristics of the signals being conveyed in the communicative process. These characteristics include method of conveyance, information contained in the signal, and the type of behavioural response induced (Brudzynski, 2005; Searcy & Nowicki, 2005).

Broadly speaking, systems of animal communication have evolved to accomplish a variety of tasks that are highly adaptive. These tasks fall in all categories of behaviour, ranging from sexual selection to aggressive encounters (Endler, 1993). A description of the sophistication and complexity of communicative behaviours found in animals extends far beyond the scope of this dissertation. It is evident that some of the most diverse and complex communication is found in mammals (Brudzynski, 2010c). Studying behaviour provides a window on the underlying neural events and processes required for their production. Communication is an ideal window in this respect as it represents a point of intersection from evolutionary, individual, and ecological pressures that ultimately provide the generation mechanism for many behaviours (Searcy & Nowicki, 2005).

The power of any proximate explanation comes from its placement in the context of the broader ultimate causes. Communication in animals is selected for because it provides the basis for a large array of advantageous behaviours (e.g., mating rituals) and because it also allows the establishment of sophisticated communicative networks among many individuals in a group (McGregor, 2005). The modality of communication is highly constrained by the environment and ecosystem the in which the organism can be most successful. For example, visual information is a poor choice at the bottom of the ocean as the amount of light is negligible (Janik, 2005). Every type of signaling has its limitations and thus for any given environment the predominant modality of signaling may be very different (e.g., visual, acoustic, olfactory, electric, etc.,). Acoustic communication is one form that appears very successful in a great variety of ecological systems, including in the ocean where marine mammals use it with great success (Janik, 2005). Because acoustic signals can be modified across a very wide spectrum they have a diverse range of application. Marine mammals often produce sounds that may travel 20-25 km or more while some terrestrial mammals can produce infrasounds (sounds between 1-20 Hz) which travel in excess of 10 km (Janik, 2005; Garstang, 2010). Thus, acoustic communication allows for networks between signalers and receivers to be established over immense distances. It can, nevertheless, also be used for networks with a much smaller (purposefully so) communication space. This constrain on active communication space may be done through the use of ultrasound frequencies (>20 kHz),which travel short distances before dissipating and are ideal for use in areas with vegetation/brush or in underground burrows (Fletcher, 2010). Thus it is not surprising to find ultrasonic vocalization used as an effective communicative behaviour in rats given that they are a prey species that often live in burrows or in dense vegetation (Brudzynski & Fletcher, 2010).

Origin of Ultrasonic Vocalization

One of the great costs associated with signaling from an individual perspective is the risk that the signal may be received by a predatory/dangerous audience. In many circumstances this risk is simply overcome by the sheer advantage conferred by the signaling itself (Endler, 1993). However, ultrasonic vocalization (USV) represents one way that the risk associated with the communicative behaviour can be lowered by reducing the probability of it being received by predators (Brudzynski & Fletcher, 2010). In doing so, USVs allow rats (and many other species) to utilize communication to establish complex social networks without taking on the maximal level of risk.

The risk of detection for a rat is a great danger; it is a prey species small in size and a predominant food source for many carnivores. Rodents have a diversity of predators in any ecosystem, each with different degrees of specializations for rodent predation. Some of these predators utilize rodents as their primary food source (specialists). Others are 'generalists' in that the number of species they consume varies based on availability (Andersson & Erlinge, 1977). The impact of these various predator types on a population of small rodents can be immense (Andersson & Erlinge, 1977; Jedrzejewski & Jedrzejewska, 1993; Sundell, 2006). Many birds of prey are nomadic specialists (e.g., owls) and will consume rodents in a given area until the number is scarce and then they will relocate to denser populations (Andersson & Erlinge, 1977). These predators are effective at killing rodents caught in the open but are generally unable to get at rodents underground inside burrows. However, other specialists (i.e. weasel) and many generalists (e.g. foxes, badgers, etc.) are able to effectively either hunt in the burrow itself or dig it up and operate as resident predators (Jedrzejewski & Jedrzejewska, 1993). More recent

investigations have found that the various predator types not only have drastic implications for the population densities of rodent species but also on behaviour and evolution of the prey species itself (Sundell, 2006).

The vulnerability of rats to so many predators permitted the evolution of a variety of behavioural and physiological adaptations. Defensive responses to predators provide one of the only avenues of survival for rodent species. It is thought most likely that USVs evolved as such a response, enabling communication that many predators cannot hear (Blanchard, Blanchard, Rodgers, & Weiss, 1990). Ultrasound communication reflects both a behavioural and physiological response to evolutionary pressures on prey species. Changes had to be made in the functioning of the larynx to produce ultrasound, but of course, changes also had to be made in auditory sensitivity to receive the calls (Brudzynski & Fletcher, 2010; Wohr & Schwarting, 2010). Cochlear responses in rodents support high frequency peaks corresponding roughly to 22 kHz and 50 kHz ranges that match the call types emitted (Brown, 1973). The genera of rat (*Rattus*) and mouse (*Mus*) both use ultrasound and are thought to have diverged 8-12 million years ago, suggesting that ultrasonic mechanisms in rodents may have a history of 20 million years or more. Though the various genera of muroid rodents all differ in how they have adapted to the pressures of selection, there are a great number of conserved survival mechanisms (Catzeflis, Aguilar, & Jaeger, 1992; Rat Genome Sequencing Project Consortium, 2004). Moreover, lacking the defensive body structures utilized by many mammals (e.g., large body size, horns, etc.), most anti-predator responses in rodents are behavioural in nature (Blanchard, Flannelly, & Blanchard, 1986).

Anti-predator behaviours can be divided into primary and secondary categories based on the nature of defense they provide to the organism (Edmund, 1974). Primary defensive

behaviours are intricately associated with the organism's baseline activities to mitigate threat from predators. These defensive behaviours include spending time underground in burrows or avoiding open spaces and only moving from cover to cover to reduce chance of predation. Conversely, secondary defensive behaviours are acute responses to the presence or threat of a predator. These defensive behaviours include escape, defensive attack, and attempts at intimidation (Edmund, 1974; Brudzynski, 2009). The production of ultrasonic calls in rats is consistent with the need of rats for an extensive defensive behavioural profile to reduce the likelihood of detection against an array of predatory threats.

Ultrasonic Vocalizations in Rats

Ultrasonic communication (above 20 kHz) is found in mammalian taxa beyond rodents (fat-tailed dwarf lemur, mouse lemur, and the bush baby all use USVs), but the Order Rodentia has a considerable number of species that utilize ultrasound (Sales, 2010). Rodents have evolved a physiological adaptation to produce USVs by distinct functions of the larynx (Brudzynski, 2005). This laryngeal adaptation provides the ability to produce two distinct sound types; one function produces audible sound (below 20 kHz) through vibration of vocal folds whereas the other produces ultrasound plausibly through a whistle-like mechanism (Roberts, 1975; Berke & Long, 2010). This whistle-like mechanism may occur through constriction of the vocal folds to create a tight orifice through which air under pressure can be pushed at such a high velocity it produces sound at ultrasonic frequencies (Roberts, 1975; Fletcher, 2010; Brudzynski & Fletcher, 2010). As a result of these two laryngeal functions, rats are able to produce sounds across much of the frequency spectrum (from audible ~2 kHz to ultrasounds of ~90 kHz). The exact mechanism of ultrasound production in rodents remains unclear. The laryngeal capacity for such production, however, is not in doubt (Brudzynski & Fletcher, 2010; Riede, 2011).

The physical characteristics of ultrasound provide some of the advantages for defense in rodents. The intensity of the sound radiating from the emitting animal decreases as a function of its distance exponentially faster the higher the frequency (Fletcher, 2010). This physical property means that ultrasonic calls propagate reduced distances compared to sonic and infra-sonic calls. Their attenuation is further increased by scattering from objects (rocks, trees, etc.,) and proximity to ground. The size of rats means that the production source of the ultrasound is indeed located close to the ground and thus the scattering and dissipation of their ultrasonic calls should be very high. This factor makes ultrasonic calls ideal for rodents as it facilitates communication as a primary defensive behaviour (Blanchard et al., 1986; Brudzynski & Fletcher, 2010). By reducing the radius of effective call propagation, the chances of alerting a predator to the presence of the calling rat are decreased. In addition, the type of environment greatly influences the physical properties of call propagation. Scattering effects on call propagation take place based on type of habitat; rocks and leafy plants may scatter the high frequency sound thereby making it harder to localize (Brudzynski & Fletcher, 2010; Fletcher, 2010). Furthermore, there is greater control with ultrasound over the directionality of the call (ultrasound is emitted as a beam) compared with audible sounds. This control of direction raises the likelihood that the call will be received by the intended audience and not inadvertently by predators (Brudzynski & Fletcher, 2010). These acoustic characteristics of ultrasound also make it very useful for use underground in a burrow. Ultrasonic calling underground would further reduce the chance of detection from predators on the ground surface, even those capable of hearing ultrasound (e.g. cats; Brudzynski & Fletcher, 2010).

Rats are highly social animals, with well-organized colonies different than many other rodents (Barnett, 1958; Blanchard & Blanchard, 1980; Brudzynski, 2009). One of the greatest

benefits of the establishment of a strong social community is the conferral of defensive advantages (Edmunds, 1974). The establishment of a colony allows for a sophisticated communication network that provides great primary and secondary defense to each individual within it. The communication of possible or detected threats is used extensively in communities of social mammals. Some communities (e.g., African meerkats) are capable of providing referential details of the threat in their calls and thus transmit such information quickly to all members of the colony (Clutton-Brock et al., 1999; McGregor, 2005).

Rats are found to produce ultrasonic 'alarm cries' in response to a predator if in the presence of conspecifics, but not if alone (Blanchard, Blanchard, Agullana, & Weiss, 1991). This conditional nature of calling indicates that the production of the ultrasonic call is directed towards others, likely serving as a defensive signal. Usually the dominant male produces this signal and it alerts all other members of the social group about the danger, but there is also evidence that high call rates may also occur in females across the colony (Blanchard, Agullana, McGee, Weiss, & Blanchard, 1992). This social organization alleviates to some degree the constant requirement for vigilance from each individual rat (with its extensive cost in time and energy) by diffusing the responsibility or centering it mostly on the dominant male (Blanchard & Blanchard, 1989). Moreover, colonies have other advantages for individuals beyond those of a simple defensive nature. For example, they provide breeding opportunities and other socially affiliative behaviours (Barnett, 1958; Blanchard & Blanchard, 1980).

In addition to alarming signals, rat ultrasounds have alternative beneficial roles such as social signals in positive contexts (Burgdorf et al., 2008). These 'social signals' may be used in a communication network to strengthen associations and enable positive interaction (McGregor, 2005). The apparent differential functions of ultrasound usage in rats suggest these calls are

associated with some kind of informational content. Unlike human communication, however, in which vocal words are said to have ‘semantic content’ (this is an anthropomorphic overreach for animal communication), animal vocalizations are thought to possess ‘semiotic content’ (Sebeok, 1965; Brudzynski, 2005). The term semiotic is a broader understanding of the symbolic value of an acoustic signal. In this context, semiotic signals possess symbolic references (to general contexts, physiological and/or psychological states, etc.,) that are derived from cues that provide some communicative value (Smith, 1965; Brudzynski, 2005). In the USVs of rats, it seems that the semiotic content refers to expression of relevant emotion (Brudzynski, 2005; Brudzynski, 2013a). Two broad classes of USVs in the adult rat are based on distinct frequency parameters: 22 kHz and 50 kHz calls. These types of calls represent two opposite emotional states and are strongly associated with alarming/aversive and hedonic/appetitive contexts, respectively (Blanchard et al., 1991; Knutson, Burgdorf, & Panksepp, 1999; Brudzynski, 2007).

It is possible that the two broad classes of rat USVs represent broadly both referential and motivational vocal signals. They may be referential in certain contexts when they convey true information which discriminates in some way an external stimulus (e.g. type of threat like as suggested for African meerkats; Clutton-Brock et al., 1999). They are also best considered as motivational because they are primarily expressing the internal state of the caller (Manser, 2010). There exists the potential that for rat USVs, the calls are often some ratio of both motivational and referential and never distinctly one or the other. Regardless, they serve to establish a communication system that is effective in both negative and positive affective circumstances (Knutson, Burgdorf, & Panksepp, 2002; Brudzynski, 2013a).

22 kHz and 50 kHz USVs in Rats

Sound productions that may occur only coincidentally with the situation in which they are evoked convey no signal information as there exists no meaningful relationship between the sound and the context. An example of this coincidence may be sneezing whereby a sound is produced and possibly received by a given audience but there is no behavioural consequence of significance for either party (i.e., no sign; Brudzynski, 2005). There is strong evidence that adult rats have a real sign communication system as many of the sounds produced appear to have a "meaningful" relationship with the context that invokes them. Emission of 22 kHz USVs represents this very well in that they are produced by rats in a colony setting in response to a fear evoking stimuli (a predator) only in the circumstance whereby an audience may benefit from the production of such a call (Blanchard & Blanchard, 1989; Blanchard et al., 1991).

Moreover, 22 kHz calls appear to produce observable behavioural reactions in the receiving conspecific (Brudzynski & Chiu, 1995) and rats show an innate preparedness to acquire defensive reactions to 22 kHz calls (Endres, Widmann, & Fendt, 2007). In addition, 50 kHz calls also show strong evidence of sign communication in playback experiments. The playback of 50 kHz calls induced approach behaviour (Wohr & Schwarting, 2007) and calling (White, Gonzales, & Barfield, 1993). Playback of male calls also appears to produce a variety of proceptive behaviours in females (McIntosh, Bartfield, & Geyer, 1978; White & Barfield, 1990). Although the calls do not appear to be innately associated with a sign function, there is a biological preparedness to condition the call with the appropriate context (aversive for 22 kHz or appetitive for 50 kHz). Thus 22 and 50 kHz signals arguably represent two separate and distinct elements of a sign communication system utilized by rats as evidenced by the evoking contexts,

behavioural responses, and even the distinguishable neuronal mechanisms (Wohr & Schwarting, 2010).

In addition to their association with a given affective context, calls are defined primarily on the basis of several acoustic parameters. 22 kHz calls have a characteristic peak frequency range (22-30 kHz) as well as a narrow bandwidth with almost no frequency modulation (Brudzynski, 2009). These calls may be very long in duration (potentially lasting thousands of milliseconds) but also some calls may be less than 100 ms (Brudzynski, 2005). On a sonogram these calls generally appear as long flat lines with little to no deviation from the relatively low peak frequency.

Subdivisions of 50 kHz USV Calls

In contrast, 50 kHz calls have distinguishable acoustic parameters that separate them from 22 kHz calls. They have a higher average (45-55 kHz) and greater general range of peak frequency (40-90 kHz) and are typically much shorter in duration (30-40 ms). Within the class of calls defined by the average peak frequency of 50 kHz, the great defining characteristic is frequency modulation (Brudzynski, 2009). Not all 50 kHz calls show any significant frequency modulation but many do, and this difference in modulation led to a subdivision of these calls into two major types: constant frequency (flat) and frequency modulated (FM) 50 kHz calls (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007; Wohr, Houx, Schwarting, & Spruijt, 2008). It is possible to further divide the flat 50 kHz calls into two subtypes based on duration (long and short), though research has often simply grouped these together (Burgdorf et al., 2008). There is the potential for further subdivision of FM 50 kHz calls based on the sonographic pattern and acoustic architecture (Wright, Gourdon, & Clarke, 2010). There is a great amount of variability in the frequency modulation of FM 50 kHz calls. This variability generates the

potential for many different subtypes to be classified, however it remains unclear if these classifications can be justified on any physiological or ethological basis.

Recent research has begun to provide more information on the differences (functional, physiological, etc.) between flat and FM 50 kHz calls (Burgdorf et al., 2008; Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009; Ciucci et al., 2009). There is little knowledge, however, about FM subtypes. This lack of knowledge has led some researchers to possibly over classify subtypes (Wright et al., 2010). Some current research appears to be focusing on the 'trill' subtype (sine-wave changes in frequency) as a biologically important call (Buck, Vendruscolo, Koob, & George, 2014; Buck et al., 2014a; Simola et al., 2014). This research suggests some physiological distinction between trill calls and other FM 50 kHz calls though much is still unclear about the various 50 kHz USV subtypes.

Neural Systems Underlying the 22 and 50 kHz Classification

There is substantial neurochemical evidence for the broad classification of 22 and 50 kHz calls based on their regulation by the ascending cholinergic and dopaminergic systems, respectively (Brudzynski, 2007). Cholinergic stimulation induces only 22 kHz vocalizations, while dopaminergic stimulation only induces 50 kHz calls. For a visual illustration of the two systems please see Figure 1.

Figure 1. Saggital Aspect of the Ascending Cholinergic and Dopaminergic Systems Underlying Ultrasonic Vocalizations in Rats.

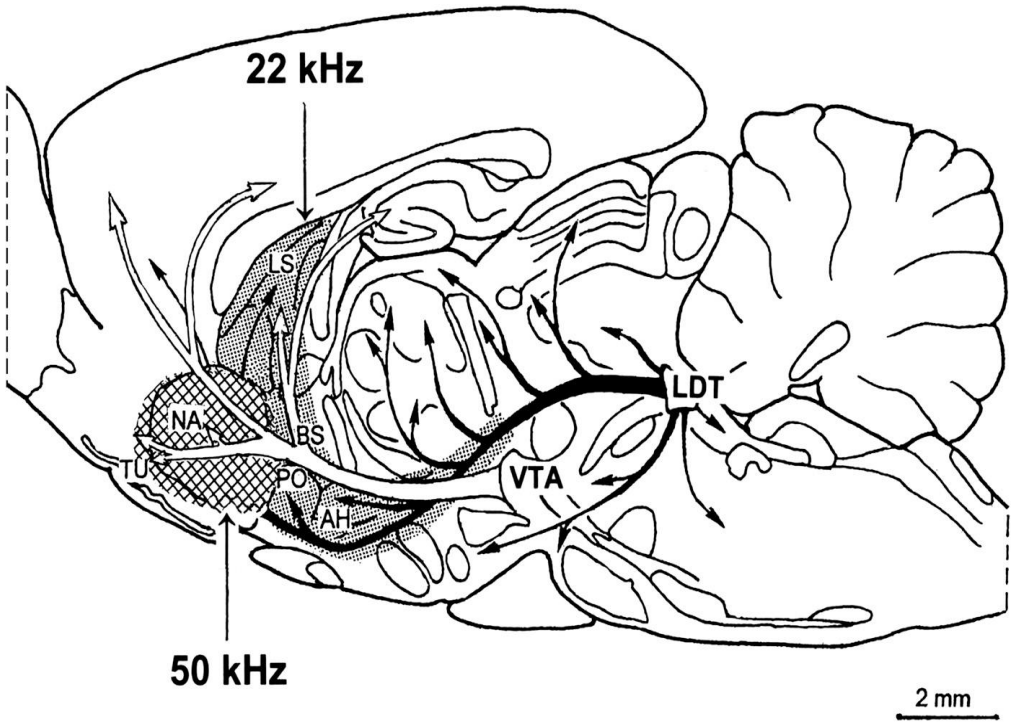

Figure 1. This figure illustrates in the rat brain the two ascending systems that underlie 22 and 50 kHz USVs. Stippled and and in black from the LDT is the cholinergic system for 22 kHz calls. In grid pattern and white from the VTA is the dopamine system for 50 kHz calls. (Figure taken from Brudzynski, 2007).

The cholinergic system underlying the production of 22 kHz calls seems to originate from the laterodorsal tegmental nucleus (LDT), which contains cholinergic neurons that project to a great number of diencephalic and forebrain target areas where they release acetylcholine (Shute & Lewis, 1967; Brudzynski, 2001). These target areas are collectively called the 'medial cholinoceptive vocalization strip' and can be pharmacologically activated with cholinomimetic agents (which act like acetylcholine). This ascending mesolimbic system was associated with initiation of defensive behaviours and vocalizations in cats (Brudzynski, 2010a).Thus there is evidence of a strong conservation of function and homologous function across species. The ascending cholinergic fibers constitute a component of the ascending reticular activating system,

which is well established to be highly important for the regulation of limbic and cortical functions (Shute & Lewis, 1967; Brudzynski, 2013b). It is the cholinergic innervation of this '22 kHz strip' from the LDT that is thought to underlie not just the production of 22 kHz calls but the negative emotional state with which they are associated (Brudzynski & Barnabi, 1996). Activity of cholinergic neurons in the LDT increases during emission of 22 kHz calls (Brudzynski, Iku, & Harness nee Savoy, 2011) and intracerebral injections (bypassing the blood brain barrier) of excitatory amino acids into the LDT elicits 22 kHz calls. This call initiation is significantly reduced with pre-treatment of scopolamine (muscarinic acetylcholine antagonist) of the target areas in the 22 kHz strip (Brudzynski & Barnabi, 1996). Moreover, intracerebral application of carbachol (an acetylcholine agonist) into this strip induces 22 kHz calling (Brudzynski & Bihari, 1990; Brudzynski, 1994). In addition, cholinergic chemostimulation of the medial cholinoceptive vocalization strip never directly induced 50 kHz calls (Brudzynski, 1994). Thus, there is abundant evidence to support the cholinergic underpinning of 22 kHz call production and that activation of this system is dissociable from production of 50 kHz calls.

In contrast to 22 kHz calls, 50 kHz USVs are associated with activity of the ascending mesolimbic dopamine system, which has projections from cell bodies located in the ventral tegmental area (VTA) in the midbrain up to many rostral areas including the nucleus accumbens (Swanson, 1982; Oades & Halliday, 1987; Burgdorf et al., 2007). Activation of this system either by electric or chemical means initiates 50 kHz calling. Application of electrical brain stimulation to various areas associated with reward (VTA, nucleus accumbens, lateral hypothalamus, ventral pallidum etc.,) induced 50 kHz calling (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Burgdorf et al., 2007). Moreover, application of pharmacological agents that increase dopamine release (i.e. amphetamine and cocaine) induced 50 kHz calling, as have agents that act directly

on postsynaptic receptors (e.g. quinpirole), showing the importance of dopamine for 50 kHz calling (Brudzynski, Komadoski, & Pierre, 2012; Wright, Dobosiewicz, & Clarke, 2013). As would be expected based on this pharmacological activation evidence, reduction of dopamine synaptic activity produces a reduction in 50 kHz calling; both 6-hydroxydopamine lesions as well as administration of dopaminergic antagonists reduced 50 kHz call production (Burgdorf et al., 2007; Ciucci et al., 2009; Wright et al., 2013). Thus, there is evidence for a paradigm of two dissociable ascending neurotransmitter systems underlying the 22 and 50 kHz call types (Brudzynski, 2013a).

Interaction of the Systems Underlying 22 and 50 kHz USVs

These ascending cholinergic and dopaminergic systems interact extensively, often in opposing ways; positive and negative states may be regulated by the mutual opposition of the two systems (Brudzynski, 2007). This mutual opposition is evidenced by the decrease in acetylcholine found in the brain of rats during self-stimulation that increases release of dopamine or treatment with amphetamine (Domino & Olds, 1972). In line with this evidence, application of cholinergic agonists reduced self-stimulation behaviour, whereas antagonists enhanced it (Newman, 1972). There is also evidence of reciprocal activation between the two systems. Immunohistochemical investigations of acetyltransferase (ChAT) labeled neurons in the LDT show synaptic interactions with tyrosine hydroxylase (TH) labeled fibers, which suggests catecholaminergic regulation (Kubota, Leung, & Vincent, 1992). This morphological evidence was supported by findings that iontophoretic application of catecholamines into the mesopontine tegmentum increased the tonic discharge of cholinergic neurons in cats. However, norepinephrine and epinephrine were found to drive this effect, whereas dopamine showed no influence (Koyama & Sakai, 2000). It is still unclear if the catecholamine innervations on LDT

cholinergic neurons may have some dopamine component. Regardless, there is better evidence of dopamine influence on cholinergic activity in the striatum. Striatal dopamine receptor activity is associated with acetylcholine release, with application of dopaminergic agonists increasing output most strongly in the shell of the nucleus accumbens. It has been suggested this role of dopamine may represent a tonic stimulatory control of cholinergic functions in the accumbens (Consolo, Caltavuturo, Colli, Recchia, & Di Chiara, 1999). The use of d-amphetamine has shown a bidirectional effect on acetylcholine release in the accumbens based on dosage and relative effects of D1 and D2 activation (Keys & Mark, 1998). Thus the dopamine influence on cholinergic function appears complicated, but the complexity goes further.

There is extensive evidence of cholinergic regulation of the mesolimbic dopamine system (for a more in depth review please see Mena-Segovia, Winn, & Bolam, 2008). Electrical stimulation of the LDT produces dopamine release in the nucleus accumbens and this effect is selectively mediated by LDT-elicited activation of cholinergic and glutamatergic receptors localized on VTA dopamine neurons (Calabresi, Lacey, & North, 1989; Forster & Blaha, 2000). Moreover, this cholinergic activation of the VTA is implicated with reward; rewarding brain stimulation is prevented if the VTA lacks these cholinergic receptors (Yeomans, Forster, & Blaha, 2001). Furthermore, blocking the cholinergic receptors on the VTA reduces dopamine efflux in the nucleus accumbens in response to morphine application (Miller, Forster, Yeomans, & Blaha, 2005; Steidl, Miller, Blaha, & Yeomans, 2011). It is possible that this cholinergic activation of the mesolimbic dopamine system represents a switching point between negative and positive states. However, it is obvious that there remains a great deal about the interactions of these systems and how they mutually regulate affective states to be elucidated as so much is still unknown (Cragg, 2006; Brudzynski, 2007). It is safe to assume that more transmitters than

dopamine and acetylcholine are likely to be implicated. For example, some evidence already suggests an important role for norepinephrine (Koyama & Sakai, 2000; Wright, Dobosiewicz, & Clarke, 2012). Regardless of the exact details, these two systems each have strong and direct associations with dissociable states of the organism (positive/negative) and are critical to understanding the organization and function of USVs in rats (Brudzynski, 2007; Brudzynski, 2013a). They both function in distinct ways to establish an internal state of affect within the organism which may consequently be expressed outwardly via emission of USVs.

Affective States

As mentioned previously, USVs as communication signals have both referential and motivational elements. As a result, they serve as an excellent example of the interaction between external stimuli/environment (referential element) and internal states/drives (motivational element). The interplay between the internal and external elements that drive communication is necessarily complex. The internal element refers to a great number of factors co-occurring in the organism at every single time point with a great degree of variability among them (health, immune function, endocrine function, etc.). The emotional systems in the mammalian brain are of primary importance in establishing an appropriate internal response to the context of the external environment (Panksepp, 2011). This establishment may be done through various means (including sensory and endocrine mechanisms etc.) but critically includes emotional or affective experiences. Thus, the organism has the internal experience of emotion based on summation of many lower-order processes (Panksepp, 2011), and this mechanism, for example, forces the organism to pay more attention to biologically important external stimuli.

To state that animals have internal experience of emotion is not an uncontroversial point as it is still debated by many (see Panksepp, 2005 for in depth review), nor can one hope at this

point in time to precisely answer the question asked by both Darwin (1872) and James (1884) "what is emotion?" Nevertheless, there is extensive evidence accrued in support of the premise that animals have internal experiences and it needs only to be tentatively assumed in order to allow significant headway be made towards further understanding what emotion really is. Its assumption enables productive predictions to be made, similar in use as many other representative concepts in psychology. These predictions may inform not only about the behaviours of animals in various novel contexts but also about the emotional functioning of humans (Panksepp, 2004). The addiction literature, for instance, is filled with striking examples of close similarity between humans and animals in their predisposition towards certain substances (Griffiths & Balster, 1979; Rose & Corrigall, 1997; Panksepp, Nocjar, Burgdorf, Panksepp, & Huber, 2004). The nature of addiction may not be identical between species. However, the strong similarity in fundamental aspects of it makes it probable that there are subjective experiences in animals and they are not wholly different from those in humans. Furthermore, the brain structures and systems that appear associated with the generation and regulation of emotion are highly conserved across mammals (Reep, Finlay, & Darlington, 2007).

Emotions serve a critical function of advantaging the organism in its interactions with the environment. They enable a value system of varying degrees of salience to be laid down on perceptions of the external world. This system allows behaviour to be organized for potential events as it creates predictive capabilities through anticipation mechanisms (Brudzynski, 2010b; Mackiewicz, Sarinopoulos, Cleven, & Nitschke, 2006). These anticipation mechanisms confer significant advantage to the organism and thus the systems responsible have been selected for and conserved across millennia of evolution (Butz & Hoffmann, 2002; Reep et al., 2007). This conservation is supported by evidence suggesting that the common ancestors of reptiles and

mammals already had established limbic structures and their subdivisions (Panksepp, 2003; Bruce & Neary, 1995). Thus, the limbic system and structures in support of its functions may be one of the many primitive elements of the mammalian brain and represents a conserved bridge between animals and humans (Damasio et al., 2000).

The advantage of emotion is found in the adaptability it provides the organism. This adaptability results from the increase in an organism's flexibility in reacting to variable stimuli. This relation with variability necessitates the existence of some variability in emotion itself. Though, as already noted, defining emotion is an immense and complicated task with no clear answer in sight, this problem may be largely circumvented by positing that the necessary variability of emotion is basically binary in its valence (positive and negative). This binary concept can be used to great effect in linking many overt behaviours to their underlying neural mechanisms (Knutson et al., 2002; Burgdorf & Panksepp, 2006; Brudzynski, 2007; Panksepp, 2011) and can serve as a foundation for resolving the complex emotional profile of humans (Davis & Panksepp, 2011). Thus the state of an organism at a given time point may be identifiable as one of these two distinguishable broad categories of affects.

Negative states are evoked by external stimuli such as the threat of present or potential danger to the organism but also internal stimuli such as abdominal pain or discomfort (Brudzynski, 2007). These states are associated with defensive patterns of behaviour (withdrawal, aversion, escape, etc,) and therefore appear centered around survival of the organism and may be described in certain instances as fear and anxiety (Panksepp, 2005; Brudzynski, 2007). In contrast, positive states are evoked in contexts associated with enhancement in security and physiological balance of the organism. The expression of positive states are often induced by social contact and affiliative behaviours (sharing food, grooming,

etc.) as these expressions are thought most critically involved in maintaining cohesiveness and stability of social groups (Brudzynski, 2007).

The communication of these affective states is an outward expression and representation of internal states governed by limbic emotive systems. The reasoning behind why these internal states should be communicated to conspecifics may lie behind the close proximity of both positive and negative states to basic survival/reproductive functions (Brudzynski, 2010b). Emotive systems are analogous to engines for behaviour; their adaptive function being derived from their flexible directive influence on overt behaviour of the organism (Panksepp, 2010). These systems can drive behaviour, and with the establishment of either a positive or negative affective state their behaviour is more likely to be adaptive. By increasing the survivability of the organism via increasing adaptability of behaviour these emotive drives were naturally selected. For negative states this adaptive trait may take the form of communication of the state to other group members allowing defensive behavioural patterns which increase survival in response to a real predatory threat (Blanchard & Blanchard, 1989; Blanchard et al., 1991). The expression of a negative state may also result from the expectation of an aversive stimulus rather than a present threat (Cuomo et al., 1992). This predictive nature of the negative state indicates the emotive system drives the expression due to the establishment of the negative affective state even if the danger is only anticipated (Brudzynski, 2007; Brudzynski, 2010b). By outward expression of a positive state, the organism may be able to stabilize the social group (calming function), procure mating opportunities, and engage in social play (Burgdorf & Panksepp, 2006; Brudzynski, 2010b; Panksepp, 2010). The likelihood of outward communication of the established affective state may depend on the intensity of the affect itself and there are certainly inhibitory mechanisms that can prevent emotional expression (Blanchard et al., 1992).

Affective states are by their very nature internal states of the organism with subjective experiences and thus at first glance may not appear amenable to scientific investigation (Panksepp, 2005). Given the relationship, however, between affective states and the patterns of behaviour they are highly involved in regulating, it is possible to use objective behavioural markers as proxies of these states (Burgdorf & Panksepp, 2006; Panksepp, 2011). With the strong association between communicative behaviour and affective states, vocalizations in rats serve as excellent indices of the internal state of the organism (Knutson et al., 2002). It has previously been noted that the 22 kHz calls are associated with the negative affective state of the rat. Specifically aversive/anxiety situations and these internal states are largely governed by the same neural mechanisms that govern initiation of call production (Brudzynski, 2001; Brudzysnki & Holland, 2005). Conversely, 50 kHz calls are associated with positive affective states (specifically appetitive/reward expectancy). 50 kHz calls thus represent a useful overt behavioural proxy for the internal affects associated with appetitive scenarios and the functions of the reward system (Burgdorf & Moskal, 2010).

50 kHz Calls and Positive Affect

Though the association of 50 kHz USVs with positive affective states is highly supported and widely recognized currently, there were a variety of hypotheses other than ones involving affect posited to explain their production. These hypotheses were alternatives to the idea that 50 kHz calls serve to index positive emotion in the rat (reviewed in Burgdorf & Moskal, 2010). They serve to illustrate that the affective association is not the only possible explanation but it is the most supported by current empirical evidence (Knutson et al., 2002). For instance, given the presumed production mechanism for USVs requiring thoracic compressions to push air through a tight orifice in a whistle-like manner (Brudzynski, 2005), it was postulated that rat USV calls

were by-products of this compression during locomotion (Blumberg, 1992). This notion would also account for the strong link between the mesolimbic dopamine system and 50 kHz call production as this system is highly associated with locomotor activity (Pijnenburg, Honig, Van der Heyden, & Van Rossum, 1976). Dopaminergic agonists that elicit 50 kHz calls also increase locomotor activity by activating this mesolimbic system (Pijnenburg, Honig, & Van Rossum, 1975; Burgdorf et al., 2001; Wright et al., 2013). In observed rats, however, only a small percentage of 50 kHz calls actually coincide with thoracic compression and a majority of calls occur unrelated to locomotor movement (Panksepp & Burgdorf, 2003; Burgdorf & Moskal, 2010). Thus, 50 kHz calls are dissociable from movement in rats and are most probably reflecting positive affect in the rat.

Given that the assumption of function for communication of positive affect is to some degree related to social contact and interaction, it should thus be expected that 50 kHz calls are strongly associated with social contexts. This expectation is highly supported by empirical findings (Brudzynski & Pniak, 2002; Burgdorf et al., 2008). Rats presented with an anesthetized same sex conspecific emitted 50 kHz calls (Blanchard, Yudko, Blanchard, & Taukulis, 1993) and called more when paired with a conspecific, whether engaged in play activity or not, than when in isolation (Knutson, Burgdorf, & Panksepp, 1998). This socially conditional nature further indicates that 50 kHz emission is unrelated to locomotor activity levels. Emission of 50 kHz calls was directly correlated with amount of appetitive play behaviour displayed during play sessions between paired rats and was observed during anticipation of play (Knutson et al., 1998). Moreover, rats emit 50 kHz calls in response to placement in a cage that has been visited by other rats. The number of 50 kHz calls emitted indexes the degree to which the cage has been exposed to other rats (Brudzynski & Pniak, 2002). Furthermore, in addition to general social

encounters, the reproductive function of 50 kHz calls is supported as they are found integral to assessing the hormonal disposition for mating of potential mates (McGinnis & Vakulenko, 2003).

Together these studies support the idea that 50 kHz calls represent a motivational state and not only the hedonic experience itself given that they are emitted highest in anticipation of rewarding social encounters (Knutson et al., 1998; Brudzynski & Pniak, 2002). This reward association does not conflict with evidence that 50 kHz calls (particularly flat calls) may function as contact calls serving to initiate contact or limit separation between group members as they may be generated by a 'seeking' system (Panksepp, 2005; Burgdorf et al., 2008; Wohr et al., 2008). It is important to note that more recent research is beginning to elucidate the apparent functional difference between flat and FM 50 kHz calls. Flat calls appear less affectively related and more socially driven (may be neutral affect), whereas FM calls appear more related to appetitive and rewarding contexts and the anticipation of them. Aggressive encounters between rats involve a greater number of flat than FM 50 kHz calls (though it should be noted the emission of calls always largely preceded the actual aggression and dropped off in rate dramatically once engaged). Moreover, heterospecific play (or 'tickling'), which involves a human researcher using their hand to mimic juvenile play with the rat, appears rewarding to rats. It also differentially elicits flat and FM 50 kHz calls, increasing rates of FM compared to flat vocalizations (Panksepp & Burgdorf, 2003; Burgdorf et al., 2008).

Thus the positive affective hypothesis appears most strongly driven by FM 50 kHz calls both in social and rewarding contexts. Rats are highly social organisms, thus many types of social interactions may be seen as highly rewarding; they show significant place preferences for the environment paired with play and also show self-administration of playback of 50 kHz USVs

(Burgdorf et al., 2008). There is, however, a variety of evidence that shows the association between 50 kHz and positive affect in experimental non-social contexts. This association strongly suggests their production signals the underlying affective state within the organism. The empirical support for this indexing is derived largely from pharmacological and electrical brain stimulation studies that show 50 kHz call emission is extensively linked with anticipation of reward and activation of reward centers in the brain (Knutson et al., 2002; Brudzynski, 2007; Burgdorf et al., 2007; Buck, Malavar, George, Koob, & Vendruscolo, 2014).

For instance, rats emit 50 kHz calls when they self-administer electrical brain stimulation to reward associated brain areas such as the VTA and lateral hypothalamus. They also emit a high number of calls in anticipation of this stimulation (Burgdorf, Knutson, & Panksepp, 2000). This evidence indicates that direct activation of the positive system in the brain with non-specific electrical stimulation can still generate 50 kHz calling (Panksepp, 2005). Moreover, this is further supported by the finding that anticipation of natural rewards (cues for food availability) generated 50 kHz calling, whereas they decreased significantly in anticipation of an aversive foot shock. This specific association with rewarding stimuli indicates that the 50 kHz calling is not resulting from general arousal but activation of specific positive emotional subsystems (Burgdorf et al., 2000; Burgdorf et al., 2007). Thus there exists evidence of multi-faceted association between 50 kHz calls and activation of the positive emotional systems of the brain. This activation, if induced via selective pharmacological means, also generates 50 kHz calling.

Peripheral administrations of drugs which activate the mesolimbic dopamine pathway (e.g. amphetamine) are associated with abuse and positive affects in humans and also unconditionally elicit 50 kHz calls (Knutson et al., 1999; Drevets et al., 2001; Wintink & Brudzynski, 2001; Wright et al., 2010). This effect in rats is blocked by administration of a

dopamine antagonist (haloperidol; Wintink & Brudzynski, 2001). Similar administration, however, of addicting drugs that are not dopaminergic (e.g. morphine) produces anticipatory 50 kHz USV production once conditioned (Buck et al., 2014; Simola, Frau, Plumitallo, & Morelli, 2014). Of particular interest is the evidence that amphetamine application differentially elicits FM over flat 50 kHz calls compared to socially induced calls (Wright et al., 2010). In humans, the increase of dopamine in the ventral striatum from amphetamine application is correlated with the experience of euphoria (Drevets et al., 2001). Moreover, rats conditionally emit more 50 kHz calls in locations associated with amphetamine or morphine administration (Knutson et al., 1999). 50 kHz emission is also lower compared to vehicle when conditioned with aversive agents such as naloxone or lithium chloride (both are well known to induce conditioned place aversion; Burgdorf, Knutson, Panksepp, & Shippenberg, 2001). There is also evidence from central administration of drugs such as amphetamine that certain reward related structures (most particularly the nucleus accumbens) are involved in 50 kHz call production (Burgdorf et al., 2001; Thompson, Leonard, & Brudzynski, 2006). Microinjections of glutamate into the anterior-hypothalamic preoptic area also induced or increased 50 kHz calling and were antagonized by haloperidol (Fu & Brudzynski, 1994; Wintink & Brudzynski, 2001). This haloperidol antagonism indicates that the increased 50 kHz calling was mediated by dopamine activity. Thus, there is extensive support for the association of 50 kHz USVs, with positive affect and dopaminergic mechanisms in the rat. 50 kHz calls are found evoked in both social and non-social contexts associated with appetitive and rewarding affect (Burgdorf et al., 2000; Burgdorf et al., 2008). The positive affect association is strongest with FM 50 kHz compared to flat 50 kHz calls and it is conceivable that the degree of modulation of calls may index the intensity of the affective state (Wright et al., 2010). That the brain systems responsible for generating the

positive affective state also appear responsible for generating 50 kHz USVs further illustrates the affective nature of these calls.

Neurophysiology underlying 50 kHz calls

In line with the positive affective hypothesis of 50 kHz USVs, the generation circuit is evidenced to be largely dependent on activity of the mesolimbic dopamine system as already noted above (Burgdorf et al., 2007). This system is referenced extensively in the literature because of its central role in reward-related learning (Spanagel & Weiss, 1999; Berridge, 2007), addiction (Pierce & Kumaresan, 2006), and affective disorders (Nestler & Carlezon Jr., 2006; Lodge & Grace, 2012). To investigate the nature of 50 kHz USVs, it is necessary to understand the brain mechanisms that give rise to them.

The midbrain dopamine system is characterized by the extensive projections from dopaminergic midbrain neurons (located in the VTA and substantia nigra pars compacta or SNc) up to a variety of rostral target areas (Swanson, 1982). The dopaminergic neurons in the midbrain represent the nuclei that are largely responsible for most dopaminergic activity in the mammalian brain (Alcaro, Huber, & Panksepp, 2007). It is important to understand the projections originating from these ventral midbrain nuclei as they are integral to concepts such as the ventral and dorsal striatum (Ikemoto, 2007). This division of the striatum is a result of the topographical organization of these projections that allows for separation of the midbrain into two functional systems.

The A9 nucleus, which functions in the nigro-striatal system, projects mostly to the dorsal striatum and has far less limbic relation than the A10 nucleus, which projects to the ventral striatum. It is this latter projection that mostly defines the mesolimbic system as the functional differences between ventral and dorsal striatum are mostly along limbic and motor

function lines (Alcaro et al., 2007; Ikemoto, 2007). Both systems are highly involved in locomotor activity (Pijnenburg et al., 1976) and project to many other structures than those in the basal ganglia (Swanson, 1982; Ikemoto, 2007).

The cytoarchitectonic features of the VTA are often continuous into the SNc with little clear division between the two (Heimer, Zahm, & Alheid, 1995). There is, however, clear division within the VTA of distinct regions (Ikemoto, 2007). The anatomical organization of these regions is evident by the topographical nature of their projections. There is a distribution of dopaminergic projections with a posteromedio-anterolateral topography at ~45 degree angle to the midline (Oades & Halliday, 1987; Ikemoto, 2007). Thus the projections into the ventral striatum from the ventral midbrain can actually be further divided based on medial or lateral aspect (for a proper review please see Ikemoto, 2007). It is the former that appears most important for common reward triggering, and amphetamine (which unconditionally elicits 50 kHz calling) acts on axon terminals within this projection system (Sellings & Clarke, 2003; Ikemoto, Qin, & Liu, 2005). The heaviest dopaminergic innervations from the midbrain neurons are found in the nucleus accumbens (Oades & Halliday, 1987). The projection systems map onto the nucleus accumbens such that the medial and lateral portions of the accumbens appear to have different functions (Ikemoto, 2007). It is thus not surprising that the nucleus accumbens is a structure with central importance in the functioning of the mesolimbic dopamine system (Westerink, Kwint, & deVries, 1996; Alcaro et al., 2007; Berridge, 2007).

The nucleus accumbens receives many limbic inputs (receiving glutamatergic inputs from hippocampus, amygdala, prefrontal cortex etc.) and is home to a complex heterogeneous array of different cell types (Heimer et al., 1995; Britt et al., 2012). These afferents have a mediolateral topography and dopamine modulates the glutamatergic input onto the medium spiny neurons of

the nucleus accumbens (Nicola, Surmeier, & Malenka, 2000; Ikemoto, 2007). These medium spiny neurons are the principal components of the nucleus accumbens (over 90% in the rat brain; Heimer et al., 1995) and have extensive efferent projections. Thus, the function of these neurons is integral to the function of the nucleus accumbens in its role of integrating limbic system input (Nicola et al., 2000). Dopamine modulates the functioning of these neurons in the nucleus accumbens and thus the inputs that originate from the VTA are integral in the structure's function. Moreover, the topography from these projections is maintained at the level of the accumbens such that the medial and lateral portions have dissociable functions in controlling motivation (Ikemoto et al., 2005). The accumbens itself is distinguishable into two sections: the core and the shell (Heimer et al., 1995) and this distinction is based on both anatomical and functional considerations (Zahm, 1999; Sellings & Clarke, 2003; Ito & Hayen, 2011). For a visual illustration of these distinct sections please see Figure 2.

Figure 2. Coronal Aspect of Calretinin Immunoreactivity in the Rat Brain Showing the Anatomical Organization of the Nucleus Accumbens.

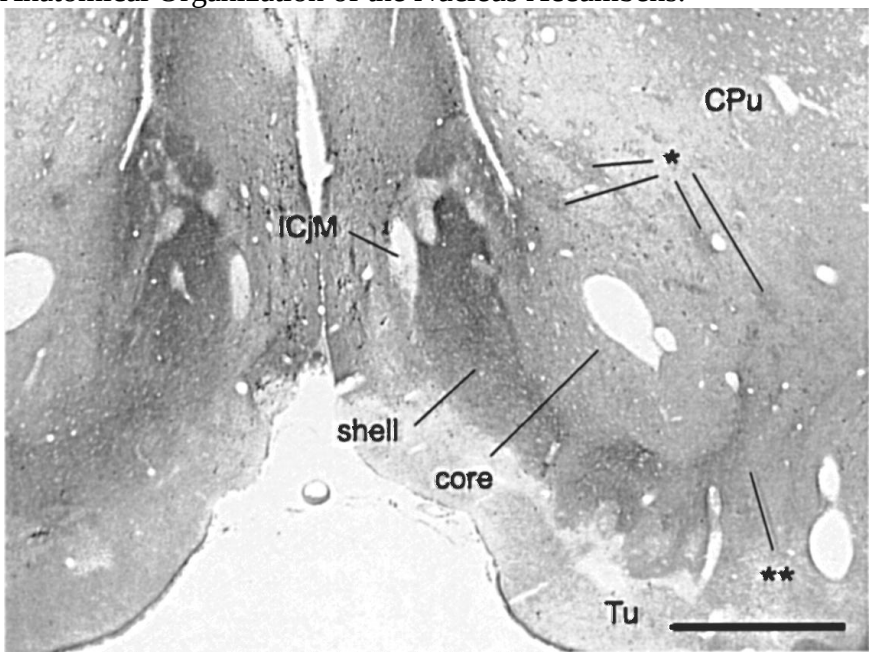

Figure 2. This figure taken from Zahm (1999) illustrates the anatomical distinction of the nucleus accumbens shell (dark) and core (light) with calretinin immunoreactivity in the rat brain. (scale bar = 1.0 mm).

The meso-ventromedial projection system from the VTA mostly innervates the ventromedial portion of the shell (Ikemoto, 2007). This portion of the shell is associated most with amphetamine reward (Sellings & Clarke, 2003) and 50 kHz USV generation (Burgdorf et al., 2001). The core the nucleus accumbens has been found strongly associated with locomotion and behavioural activation but does not appear to strongly support 50 kHz call generation (Thompson et al., 2006).

Thus, the mesolimbic dopamine system underlies 50 kHz call generation as evidenced by pharmacological, electrical brain stimulation and lesion experiments. These experiments outline the key areas of investigatory interest by indicating the physiological variables associated with 50 kHz calling (Burgdorf et al., 2007; Brudzynski et al., 2012). One of these physiological variables which is of interest is the role of dopamine activity in the nucleus accumbens as it appears to be an integrator of limbic information, and is central to the mesolimbic systems functioning (Ikemoto, 2007; Brudzynski, 2013a).

Current Research and Rationale

The strong association between 50 kHz USVs and the mesolimbic dopamine system makes it likely that the nucleus accumbens, which is a main terminal for much of the A10 nuclei's projections, is a critical initiator of calls (Brudzynski, 2013a). It has a central importance in the activity of this dopamine system (for full review see Ikemoto & Panksepp, 1999). Moreover, much of the literature regarding activation of positive affect with induction of 50 kHz calling focuses on this structure (Burgdorf et al., 2001; Knutson et al., 2002; Brudzynski et al., 2012). Although a variety of non-dopaminergic mechanisms exist to establish a positive affective

state (opiate or GABA receptors in limbic structures, neuropeptide regulation etc.), 50 kHz calls have been thus far most associated with dopamine activity in the nucleus accumbens (Burgdorf & Panksepp, 2006; Thompson et al., 2006; Burgdorf et al., 2007). Moreover, there appears to be important fundamental differences within the 50 kHz call category between flat and FM subtypes (Burgdorf et al., 2008). It is the latter (FM 50 kHz calls) that seem to be the best index of positive affect; generation of these calls was best localized to ventromedial portion of the accumbens shell, and could be directly elicited by intraaccumbens application of amphetamine (Thompson et al., 2006; Wright et al., 2013). Within this FM sub-category, Wright and colleagues (2010) identified a wide variety of potential subtypes of 50 kHz calls based on their frequency modulation characteristics. Though very little is known about the significance of these subtypes, the idea that rats exhibit a 'call profile' was posited. This concept holds that the proportions of 50 kHz call subtypes emitted by a given rat under a given circumstance are relatively stable.

Furthermore, the studies showed that this call profile may be affected by type of induction of calls (social versus pharmacological) and found evidence that amphetamine specifically seems to increase the proportion of trill calls. Call induction was via peripheral injection of the drug (intraperitoneally) and showed increases in the trill proportion compared to vehicle and social contact evoked calls. There are, however, possible alternative explanations for the generation of these subtypes (i.e., locomotor or motor by-product) and that they do not truly represent different semiotic signals. One critical step towards understanding them is to determine if they can be individually attached to the neuroanatomical foundation that supports the affective hypothesis of 50 kHz calls. Namely, that experimental change to the mesolimbic dopamine system should change the 'call profile' and thus change the proportions of these USV subtypes.

The purpose of the current research was to investigate these potential FM subtypes of 50 kHz calls on the basis that modulation of frequency itself may be of critical importance in indexing affect. The role of the nucleus accumbens and effects of injection route on the generation of FM call subtypes was investigated in a series of experiments utilizing amphetamine induced calling. Experiment #1 sought to investigate whether systemic subcutaneous injections of amphetamine would induce a significant increase in proportions of trill and step calls compared to control. Experiment #2 sought to determine if this effect of amphetamine on call profile was driven by action in the nucleus accumbens. Experiment #3 investigated the role of the nucleus accumbens in the generation of this amphetamine-induced call profile by microinjections of procaine into the shell (reversible lesion) combined with subcutaneous injections of amphetamine. The purpose of the procaine was to prevent conduction of neural activity from the nucleus accumbens shell to its downstream targets, thereby producing an effective blockade of the nucleus accumbens shell output.

There were 3 hypotheses generated for these experiments: 1) Amphetamine will induce a shift in call profile such that proportions of trill and step calls will be increased compared to vehicle and amphetamine induced calls will have greater bandwidth compared to vehicle. 2) Direct application of amphetamine into the accumbens will induce 50 kHz calling, with proportions of call subtypes and bandwidth not differing from those induced by systemic application of amphetamine. 3) Amphetamine-induced calls with procaine blockade of the nucleus accumbens will have reduced proportion of trill and step calls as well as reduced bandwidth compared to amphetamine-induced calls without procaine blockade.

Methods

Animal Subjects

Twenty-four adult male Long Evans rats (procured from Charles River Laboratories, Quebec) with body weights between 300-500 g were used in this study. Once at Brock University, the animals were housed (12 in Brock University's Mammal Facility and 12 in The Cairns Family Bioscience Research Complex Animal Facility) in translucent polycarbonate containers that were 560 mm x 250 mm x 195 mm in size. The animals were given at least 4 days to acclimate to the new facility. They were housed with a PVC plastic tube for hiding, aspen block of wood, and paper towels in their container to maintain a comfortable and enriched environment. Two types of bedding were used throughout the study (12 rats had dust-free corn cob bedding, 12 had Pure-o'Cel paper bedding from Andersons Lab Bedding, Ohio, USA). The change in bedding was necessitated by the moving of the animal facility to a new building. Food (standard chow from Harlan Laboratories, Wisconsin, USA) and water were available *ad libitum* and both light and temperature were controlled (light cycle was 12:12 hours with light on at 7 am and room temperature 21 ± 1ºC). All rats were housed in pairs during initial acclimation. Rats receiving only subcutaneous injections remained housed in pairs, and rats receiving stereotaxic surgery were housed singly post-operation.

All experimental procedures throughout study were conducted in accordance with the Canadian Council on Animal Care, approved by the Brock University Animal Care and Use Committee (AUPP: 12-09-05) and with veterinary care available at all times. Surgical procedures and post-operative care of the animals was always done with the supervision of an appointed veterinarian. No procedures took place on rats that had received surgery until at least 5

days post-operation and only on healthy rats. All rats maintained good health and showed species-typical behaviour throughout the study.

Stereotaxic Surgeries

Twelve rats were implanted stereotaxically with chronic cannulae in the brain for intracerebral injections. This implantation allows for the microinjection of a given substance into the precise structure under investigation. In preparation for the surgical procedure the rats were anesthetised with isoflurane (5% induction, 2% maintenance) and mounted on a stereotaxic frame (David Kopf Instruments, CA, USA). Upon achievement of appropriate anesthetic sleep depth, a dose of antibiotic (trimethoprim and sulfadiazine 1:5 at a dose of 240 mg/kg) was administered to prevent any infection and a dose of analgesic (meloxicam 2 mg/kg) was administered to prevent pain and discomfort upon waking. The surgical site was prepared by application of a 7% iodine scrub solution, followed by 70% isopropanol, and finally locally treated with 10% iodine.

Following incision, 23 gauge stainless steel guide cannulae (constructed from syringe needles, Becton Dickinson & Co., NJ, USA) were implanted bilaterally into the nucleus accumbens shell (10.2 mm anterior, 0.8 mm lateral, and 7.6 mm ventral from lambda) according to coordinates from a stereotaxic atlas (Paxinos & Watson, 1986). The guide cannulae were secured to the skull using small stainless steel screws and dental acrylic (DenPlus, Longueuil, QC). Removable stainless steel wire plug pins were used to block the opening of the guide cannulae. After surgery rats were placed in a recovery cage and were given wet chow and a nutritional supplement (DietGel Boost from ClearH_20, Portland, USA) and monitored until recovery.

Injection Procedure

Rats were regularly handled and received mock injections to habituate them to the procedure. Across all experiments rats received up to a maximum of 5 intraaccumbens and 5 subcutaneous injections per rat.

Rats in experiment 1 were removed from their paired cage and brought to the acoustic recording room where they received a subcutaneous injection of either amphetamine or saline and then were placed singly into a waiting cage for ten minutes before placement into the recording cage for ten minutes. There were 4 days between injections.

Rats in experiment #2 were removed from their individual housing and brought to the recording room and were injected either subcutaneously or intracerebrally with amphetamine (s.c./intracerebral route counter balanced within subjects). Recordings were again made after a ten minute waiting period after subcutaneous injections, however they were made directly after the bilateral intraaccumbens injections. There were always 4 days between each injection in this set.

Rats in experiment #3 were removed from their individual housing and brought to the recording room and were injected intracerebrally with either procaine or saline (procaine/saline was counter balanced within subjects), which was immediately followed by subcutaneous amphetamine. Recordings were made ten minutes after the subcutaneous amphetamine injection. There were always 4 days between each injection in this set.

Thus rats in both experiment #2 and #3 had double injections each injection day. Experiment #2 and #3 were performed on the same rats (experiment #3 followed #2) and were subsequently followed by control injections in the same rats ,which involved intracerebral application of either procaine or saline (procaine/saline again was counter balanced within

subjects) followed by subcutaneous injections of saline. Recordings were made ten minutes after the subcutaneous injections of saline. There were always 4 days between each injection in this control set.

Drug Injections

d-amphetamine hemisulfate (Sigma-Aldrich, QC) was dissolved in saline (0.9% NaCl, Baxter Corp, ON) to a dose of 1.5 mg/kg in 0.2 ml for experiment #1 subcutaneous injections, 2 mg/kg in 0.2 ml for experiment #2 subcutaneous injections, and 7 µg/0.6 µl (3.5 µg/0.3 µl on each side) for all bilateral intracerebral microinjections of amphetamine Dosage was adjusted for weight of rat using average of group for a given injection day. The dosages of amphetamine chosen for both subcutaneous and intracerebral microinjections have been used extensively in the literature to evoke 50 kHz USVs in male rats (Burgdorf et al., 2001; Wintink & Brudzynski, 2001; Thompson et al., 2006).

Procaine hydrochloride (Sigma Chemical Co., MO, USA) was dissolved in saline to a dose of 50 µg/1 µl (25 µg/0.5 µl for each side) for experiment #3 and control bilateral intracerebral microinjections. The procaine solution had a pH of 6.8. The dose for the procaine has been used in previous literature investigating the role of the accumbens shell and its involvement in locomotor activity by inactivation with procaine (Ikemoto & Witkin, 2003). This dosage is decidedly moderate to small, as procaine usage ranges widely in doses (Brudzynski & Mogenson, 1985; Morency & Beninger, 1986; Morency, Stewart, & Beninger, 1987). Control bilateral microinjections with vehicle were done with 1 µl (0.5 µl per side) volume. The drug procaine was chosen to cause reversible blockade of the nucleus accumbens shell because as a local anesthetic it blocks voltage-gated sodium channel activity (Creveling et al., 1990) thereby disrupting action potential propagation and nerve conduction (Franz & Perry, 1974). This

property in addition with its actions similar to cocaine (though significantly less potent) on blocking dopamine uptake give it the capability to allow amphetamine to increase dopaminergic activity while presumably blocking output from the accumbens shell (Woodward, Compton, Balster, & Martin, 1995). This trait of procaine greatly contrasts electrolytic lesions, which irreversibly destroy tissue and cannot have any subsequent injections. Thus in principle, the role of the nucleus accumbens shell in amphetamine-induced modulation of 50 kHz call profile can be investigated while the general functions of the mesolimbic dopamine system and dopamine activity in the shell remain intact.

Rats were gently restrained during all injections and had been habituated to the handling for 4 days beforehand. For microinjections the plug pins were removed and sterile injection cannulae were placed into the guide cannulae. 70% alcohol was used to sterilize openings of injection cannulae, opening of the guide cannulae, and plug pins. The intracerebral application of the drug was done via an injecting cannula (30 gauge, Small Parts Inc., Florida, USA) connected with Intramedic polyethylene tubing (PE–10, internal diameter of 1.57 mm, Becton-Dickinson & Co., Mississauga, ON) to a CR-700 constant rate syringe (Hamilton Company, Nevada, USA). Injecting cannula was inserted into the guide cannula so that it protruded 1 mm below the lowest end of the guide cannula.

Ultrasonic Vocalization Recordings

All recordings were ten minutes in length and were conducted in a small (220 mm x 200 mm x 190 mm) polycarbonate cage. The recording was made with a CM16/CPA condenser microphone (working range of 10 – 250 kHz, Avisoft Bioacoustics, Berlin, Germany) that was located on a wire lid approximately 20 cm away from the animal. Every rat received a fresh and clean recording cage to prevent exposure to odours from other rats. Avisoft Bioacoustics

software (Avisoft Recorder NIDAQMX) was used to record the USVs and enabled storage on a computer hard drive and DVD disc for analysis.

Acoustic Analysis

Sonographic characteristics of the calls were analysed off-line using Avisoft SASlab-Pro program. For every call in any condition there was a determination of its general parameters and a classification of its type. General call parameters included peak frequency (frequency of the call that occupies the greatest power level measured in kHz in the power spectrum), duration (length of the call measured in milliseconds), and bandwidth (difference between the highest and lowest frequency within the call measured in Hz). The calculation of these parameters was used to classify the call into a subtype of 50 kHz USVs (22 kHz calls were rare and were not included in analysis). Subtypes were chosen and characterized in a similar manner to categories described in the literature (Wright et al., 2010; Brudzynski, 2013). Not all 14 subtypes used by Wright and colleagues (2010) were utilized but 5 distinct major types were derived from this variety of categories. This reduction in subtypes was chosen to conserve statistical power. For a description of each subtype used, see Table 1.

Table 1

Simplified description of typical characteristics of each of the 5 call subtypes used as 50 kHz USV classifications.

50 kHz Subtype Name	Peak Frequency (kHz)	Duration (ms)	Bandwidth (Hz)	Visible Sonographic Pattern
Flat	50	Typically short (5 – 12 ms) or long (>30 ms)	Narrow, < 6000	Short dot or long line
Trill	50	Typically long (>30 ms)	Very broad, > 15,000	Rhythmic waves of ups and downs
Step	50	Medium (15 – 30 ms)	Broad, > 8000	Stair steps either up or down
Ramp	50	Medium (15 – 30 ms)	Medium, > 6000	A continuously ascending or descending ramp
Complex	50	Highly variable (10 – 40 ms)	Variable, ~5000 to 15,000	Variable changes in ascending/descending with no constant pattern

For experiment #1 all files were analyzed across the entire 10 minute session. The amphetamine files had so many calls (>200) that in order to make comparisons with the saline control files three 30 call samples were taken from each file (from beginning, middle, and end of the recording). These samples were collapsed together to represent the call profile for each of the amphetamine files. All calls throughout the entire 10 minute session were used for the saline files due to the lower number of calls emitted in this condition. For experiments #2, #3, and the control set, all files were analyzed across the entire 10 minute sessions with all calls in the files being included. For a sonographic representation of the 5 call subtypes used to classify with exemplary sonograms please see Figure 3.

Figure 3. Exemplary Sonograms of 50 kHz Ultrasonic Vocalization Subtypes Utilized in Current Study.

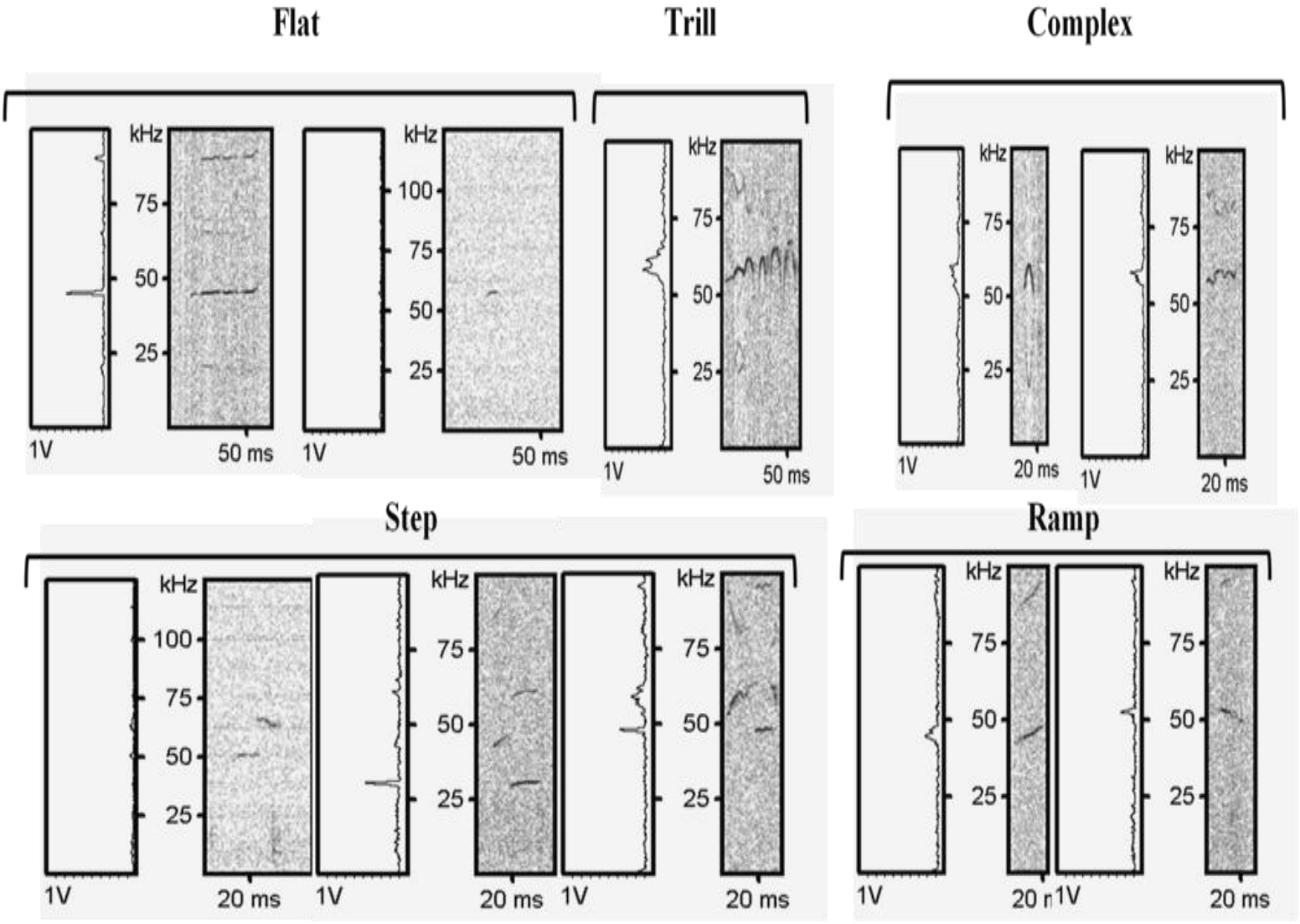

Figure 3: Representative calls for the 50 kHz USV subtype classifications shown on exemplary sonograms enabling visualization of the acoustic pattern. Each grey box is the sonogram with call image. Multiple appearances of the call are harmonics of the emitted call. The power spectrum is found in the box to the left of each call's sonogram.

Histological Procedure

Upon completion of all injections for experiment #2, #3 and control sets, the placement of the cannulae in the rat brains were determined. Before sacrifice, 0.5 µl of Indian ink was injected into each cannula to increase the accuracy of locating the injection site. The animals were sacrificed with an over-dose of sodium pentobarbital (240 mg/ml at ~120 mg/rat) to deeply anesthetise, then saline was perfused transcardially to fix the brain for extraction. After extraction, the brains were placed in a 10% formalin solution to fix them for at least 48 hours before sectioning. For sectioning, the brains were each blocked, mounted on a freezing microtome (Hacker Instruments, NJ, USA) and sectioned coronally (approximately 40 µm slices). The sectioned slices were placed on polylysine-coated microscope slides and air dried for at least 24 hours in preparation for staining.

The staining procedure used was a modified version of Nissl staining, the Rucker-Koithan method (Windle, Rhines, & Rankin, 1943; Skinner, 1971). A thionin solution was used to stain the slides (3 minutes of exposure). After the thionin, the slides were placed in alcohol baths for three stages of increasing concentrations to differentiate (4 minutes of exposure each). After differentiation, the slides were cleared in xylene for 5 minutes. Cover-slipping of the stained slides was done with Permount glue (Fisher Scientific, NJ, USA). This process allowed for the site of injection to be visualized under a light microscope and comapred to a coronal section of a stereotaxic atlas (Paxinos & Watson, 1986).

Statistical Analyses

To compare the proportions of various 50 kHz call subtypes across conditions, the number of observations of each call subtype was converted to a percent for that individual rat. These percents were then averaged to represent the average proportion of that call subtype in a

given condition (e.g. subcutaneous amphetamine in experiment 1). The proportions of subtypes were compared in a percent distribution and thus vulnerable to biasing influence from the inter-individual variability of calling (some rats had far more calls made than others, this leads to disproportionate percentages). To attempt to attenuate this influence, all subtype data for experiments 1-3 underwent linear transformations according to the formula $X_{new} = bX_{old} + a$ (where a and $b = 1$). This transformation ensured there was a minimum of a 10% baseline for each rat's call subtype.

To investigate if two conditions differed in their 50 kHz USV subtype proportions while taking all subtypes of calls into account, a Friedman's ANOVA (non-parametric test analogous to the repeated-measures ANOVA) was used. To compare two means across any two conditions the Wilcoxon matched-pairs signed-ranks test (a non-parametric procedure analogous to the paired-samples t-test) was used. Non-parametric procedures were used for all statistical analyses because of the nature of the data. To reduce the number of comparisons investigated, of the general parameters, only bandwidth was investigated across the different experiments. This parameter was chosen because it is a strong index of frequency modulation and serves to investigate general frequency modulation differences between two conditions without taking into account subtypes.

With multiple comparisons made to investigate the differences in proportions, there was a concern of inflating type I error. The concept of controlling false discovery rate has been posited as an alternative method to the multiple comparison multiplicity problem and works very well with non-parametric procedures (Keselman, Cribbie, & Holland, 1999). The Benjamini and Hochberg's Linear Step up Procedure was used to control the false discovery rate and thus the chance of making a type I error (Benjamini & Hochberg, 1995). Most statistical calculations

were conducted using IBM SPSS Statistics (IBM, New York, USA), all others (averages, standard errors, and false discovery rate) were carried out with Microsoft Excel or on paper.

Results

Injection Site Localization

The ventromedial portion of the nucleus accumbens shell was the target area for microinjections in experiments #2 and #3. Nine rats had injection sites localized in this target area (3 rats had no injection site in the shell). The localizations found in the accumbens shell are displayed in Figure 4. For a display of injection sites localized outside the nucleus accumbens shell please see Figure 5.

Figure 4. Localizations of Injection Sites in the Nucleus Accumbens Shell.

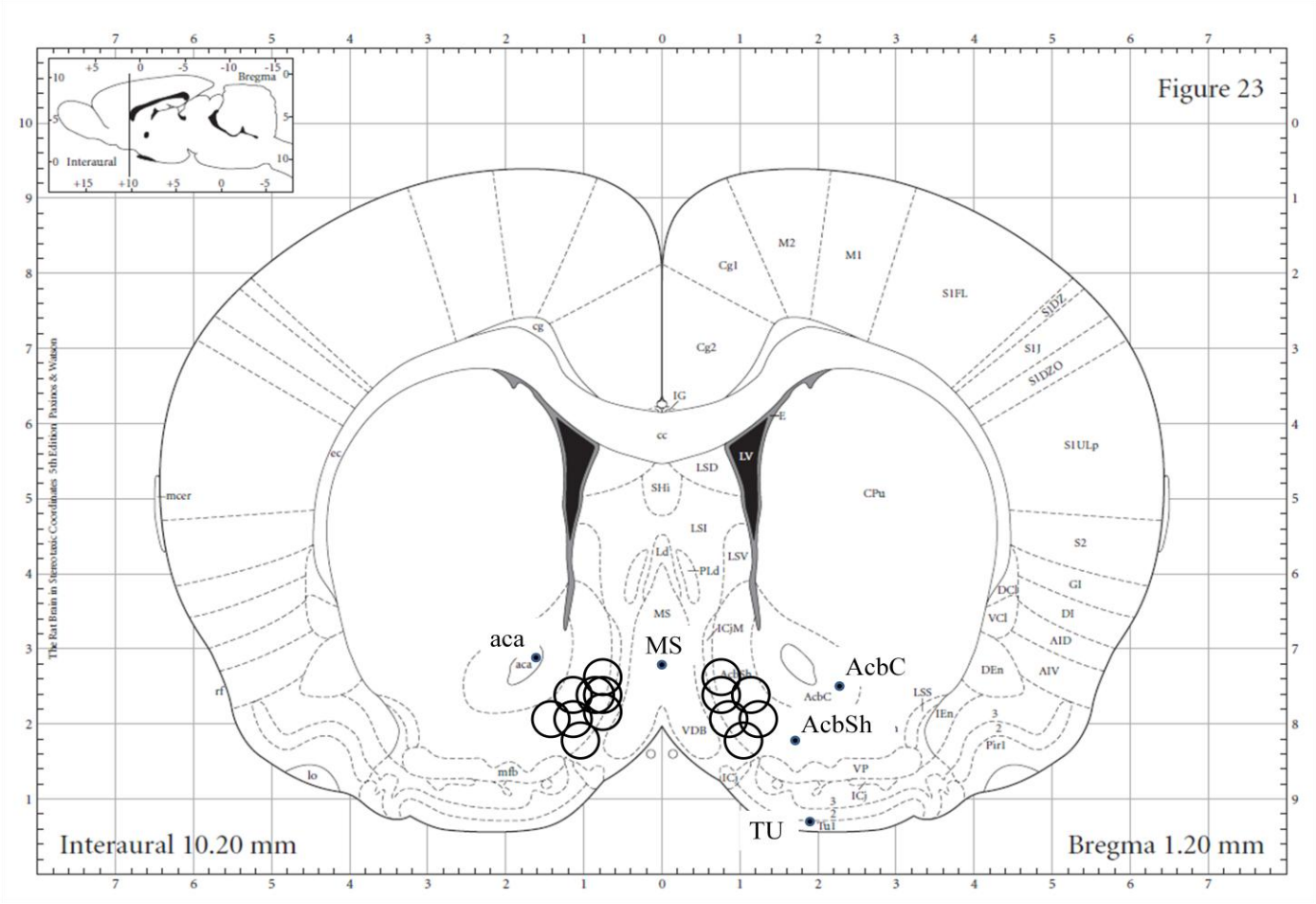

Figure 4. Injection sites for bilateral nucleus accumbens shell cannulae implantations for experiments #2 and #3 localized on a coronal section of the rat brain 10.2 mm from the interaural zero plane (Paxinos & Watson, 2005). Rostro-caudal relationship not depicted. Diameters of the circles represent the outer diameter of the injection cannulae. Major landmark areas have

enlarged abbreviations. Abbreviations: aca: anterior commissure, AcbC: nucleus accumbens core, AcbSh: nucleus accumbens shell, ICj: islands of Calleja, ICjM: islands of Calleja major island, mfb: medial forebrain bundle, MS: medial septum, Tu: olfactory tubercle. Scale at the bottom in millimetres (one division = 0.2 mm).

Figure 5. Localization of Injection Sites outside the Nucleus Accumbens Shell.

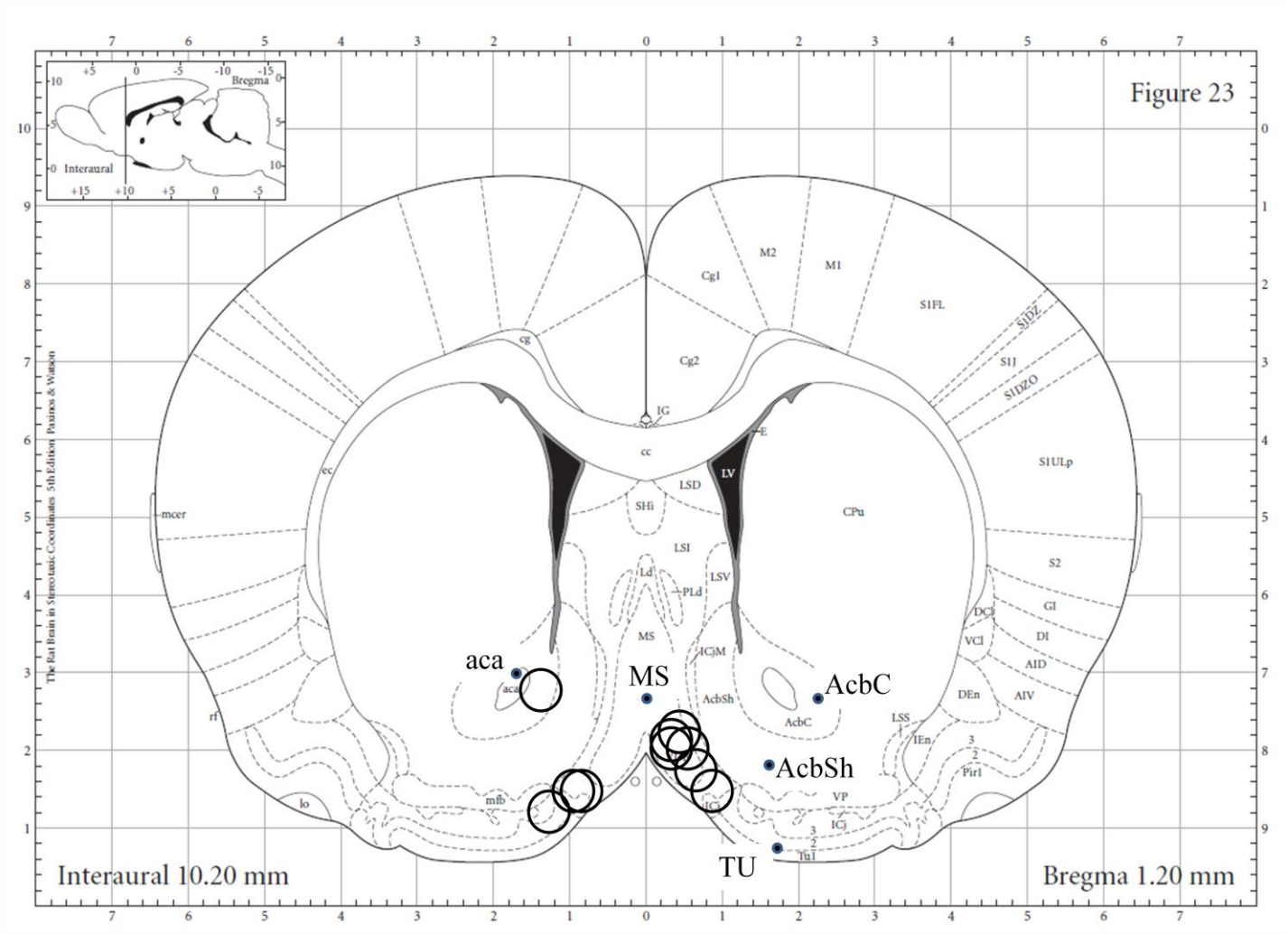

Figure 5. Injection sites for cannulae implantations localized outside of the nucleus accumbens shell for experiments #2 and #3 on a coronal section of the rat brain 10.2 mm from the interaural zero plane (Paxinos & Watson, 2005). See legend to figure 3 for further explanations.

Experiment #1

As stated in Methods, experiment #1 involved subcutaneous injections of either amphetamine or saline to determine the effect of systemic amphetamine administration on 50

kHz USV call profile. Upon examination of the saline and amphetamine files, it was evident that amphetamine resulted in a greater number of 50 kHz calling than saline alone. Thus it was necessary to take only 90 calls (from 3 time samples) from each amphetamine file in order to compare with the lower number of calls emitted on average in the saline condition.

A non-parametric Friedman's ANOVA was conducted on the parameter of bandwidth across the three samples taken from each file to determine if they fairly represented the entire recording sessions. Bandwidth was used as it is a strong indicator of frequency modulation throughout the file. The components did not differ significantly for the general acoustic parameter of bandwidth (X^2 (2) = 2.93, p = .231). Thus the components were collapsed and used to represent the recording session for each amphetamine file.

Some rats were eliminated from analyses (statistical outliers) because they emitted an insufficient number of calls (n = 4) in either the saline condition or amphetamine condition, which made comparisons between the two unfeasible. General acoustic parameters were calculated for each USV found in the recording session for each rat across both conditions. See Table 2 for descriptive statistics.

Table 2

Descriptive statistics for experiment #1 (n = 8) general acoustic parameters of 50 kHz USVs for both subcutaneous injection conditions

Condition	Number of Calls Emitted		Peak Frequency (kHz)		Duration (ms)		Bandwidth (Hz)	
	M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
Saline	25	13	51.23	4	21.6	3	6421	1006
Amphetamine	90	0	52.96	4	27.6	5	9411	1630

A Wilcoxon matched-pairs signed-ranks test was performed across the saline-amphetamine conditions for the parameter of bandwidth to determine if the two groups differed

in the amount of frequency modulation. Bandwidth serves as a non-subtype specific variable to compare the frequency modulation across conditions. Calls after administration of amphetamine had significantly higher average bandwidth (Z = -2.52, p = .012) than did those after administration of saline vehicle. This higher bandwidth indicates that the amphetamine-induced USVs had on average a greater difference between their highest and lowest frequencies compared to saline calls. This finding suggests increased frequency modulation in the amphetamine injection condition (see Figure 6).

Figure 6. Average Bandwidth of 50 kHz Calls Comparison for Experiment #1.

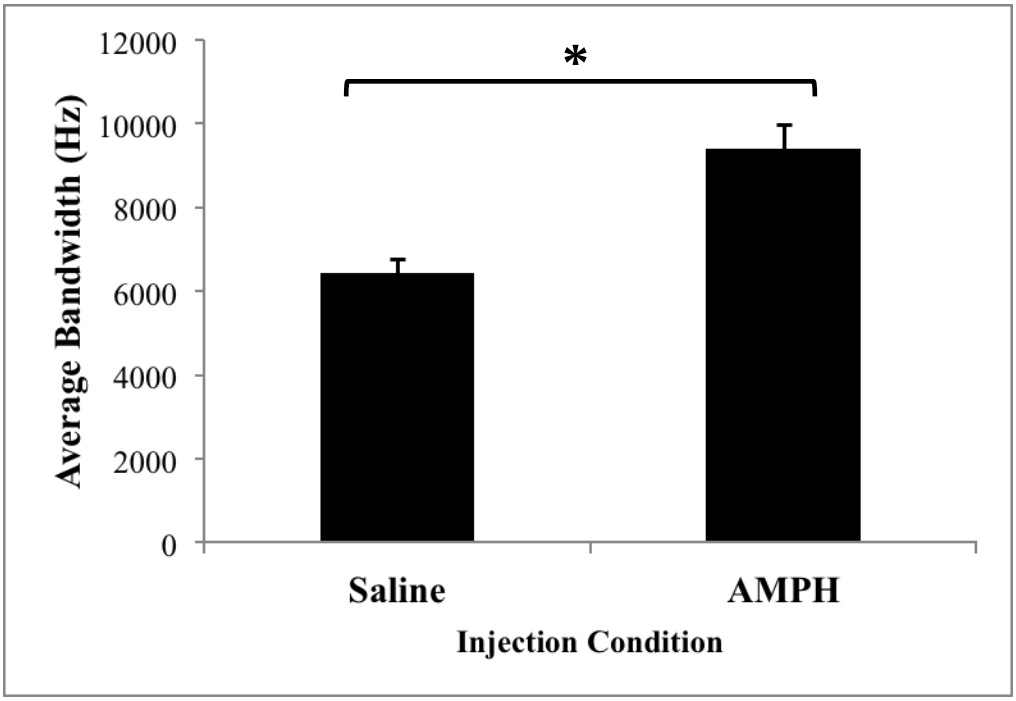

Figure 6. Average bandwidth comparison between systemic subcutaneous saline and amphetamine injection conditions which indicates a significantly greater average bandwidth in amphetamine condition (AMPH). This suggests amphetamine increases frequency modulation of 50 kHz USVs compared to saline. Error bars represent the S.E.M.
* $p < .05$

To investigate whether the subcutaneous amphetamine-induced 50 kHz USVs replicated the preferential increase in trill and step calls over saline found in the literature, the 50 kHz call

subtypes were compared across the two conditions (see Table 3 for descriptive statistics of raw number of calls).

Table 3

Average number of calls (raw) of each 50 kHz USV subtype emitted in both subcutaneous injection condition.

Condition	Flat	Trill	Step	Ramp	Complex
Saline	11	2	3	4	10
Amphetamine	33	12	18	7	25

As can be seen from Table 3 the amphetamine condition elicited a greater number of calls, however, these calls were converted to percents of each rat's total calls within each injection condition in order to compare proportions of subtypes between conditions (see 'statistical analyses' in Methods). A Friedman's ANOVA was performed on all 5 subtypes to determine if there was a difference in the proportions of subtypes emitted in the two injection conditions. A significant difference was found (X^2 (9) = 48.204, $p < .001$) and thus post hoc Wilcoxon matched-pairs signed-ranks tests were performed on specific subtypes of interest. Included in the analysis was trill and step as these were the FM 50 kHz call subtypes previously shown to be preferentially increased by amphetamine (Wright et al., 2010; Wright et al., 2013). Flat 50 kHz calls, however, were also included to account for the possibility that a change in the proportion of flat and not FM 50 kHz could be driving the difference between the two conditions. Given the lack of evidence suggesting amphetamine modulates proportions of ramp and complex calls (Wright et al., 2010) these subtypes were not included in the analyses to conserve statistical power. The amphetamine condition was found to have a significantly greater proportion of trill calls (Z = -2.51, p = .012) and step calls (Z = -2.10, p = .036). This finding indicates that FM 50 kHz subtypes were driving the difference between the conditions as there

was no significant difference found between subcutaneous saline and amphetamine for proportions of flat 50 kHz calls (Z = -1.12, p = .263). For a graphical representation of the proportions of all subtypes across the two injection conditions please see Figure 7.

Figure 7. Comparison of Proportions of Subtypes Between Conditions for Experiment #1.

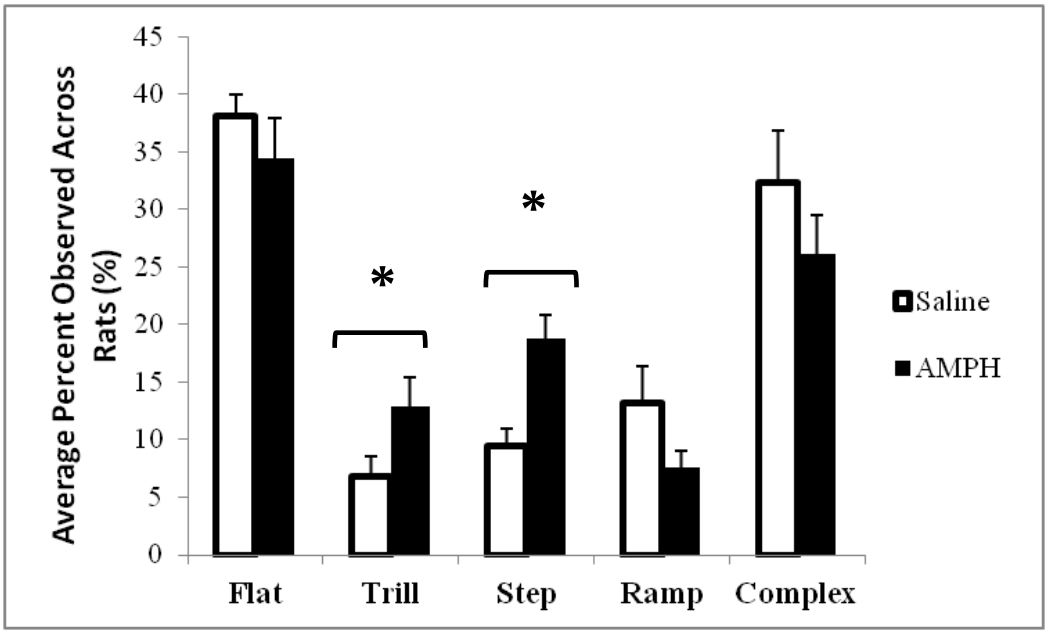

Figure 7. Proportions of 50 kHz USV subtypes compared across subcutaneous injection conditions for n = 8 rats. This figure illustrates that the difference found between the two conditions is likely due to the increase in trill and step calls relative to other call subtypes in the amphetamine condition (AMPH) compared to saline. Error bars represent the S.E.M. (100% = all calls for one condition).
* $p < .05$

Experiment #2

As stated in Methods, experiment #2 involved injections designed to determine if the routes of injection influenced the effect of amphetamine on the 50 kHz USV call profile. Amphetamine was injected either directly to the nucleus accumbens via intraaccumbal microinjections or systemically via subcutaneous injections.

After cannulae localization, three rats were removed from analyses because the injection site was outside of the nucleus accumbens shell (see 'Injection site localization' in Results). Moreover, one rat was removed because it made too few calls across the conditions. Thus, a total of four rats were excluded from the analyses in experiment #2 leaving an n of 8. General acoustic parameters were calculated for each USV found in the recording session for each rat across both conditions. See Table 4 for descriptive statistics.

Table 4

Descriptive statistics for experiment #2 (n = 8) general acoustic parameters of 50 kHz USVs for both intraaccumbens and subcutaneous amphetamine injections

Condition	Number of Calls Emitted		Peak Frequency (kHz)		Duration (ms)		Bandwidth (Hz)	
	M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
Intraaccumbens	13	10	49.42	12	14.6	10	5558	2247
Subcutaneous	31	29	55.04	5.7	24.9	9	9254	3105

To investigate the possible differences between routes of application in inducing general frequency modulation independent of subtype proportions the general acoustic parameter of bandwidth was investigated between the two conditions. Subcutaneous amphetamine-induced 50 kHz USVs were found to have a higher average bandwidth compared to intraaccumbens amphetamine-induced calls ($Z = -2.52$, $p = .012$). This finding suggests that the systemic administration of amphetamine induced greater frequency modulation of 50 kHz USVs when compared to direct application into the accumbens shell (see Figure 8).

Figure 8. Average Bandwidth of 50 kHz Calls Comparison for Experiment #2.

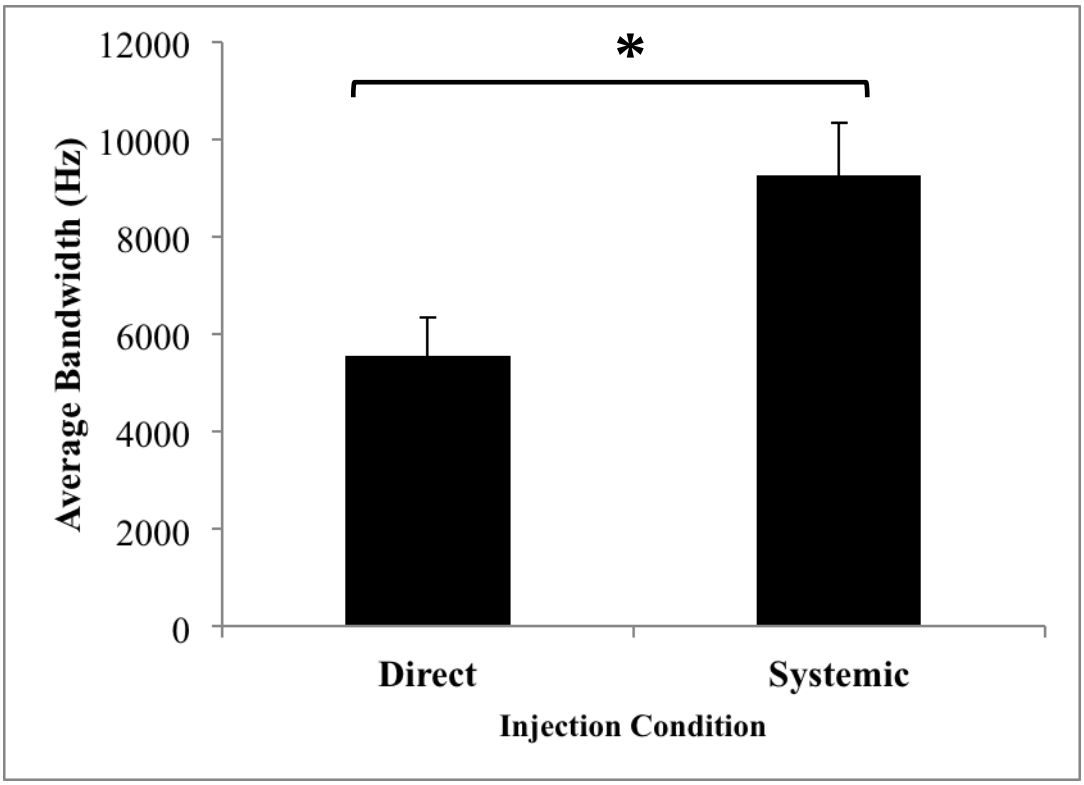

Figure 8. Average bandwidth comparison between intraaccumbens (direct) amphetamine and subcutaneous amphetamine (systemic) injection conditions which indicates a significantly greater average bandwidth in the subcutaneous amphetamine condition (systemic application). This suggests amphetamine increases frequency modulation of 50 kHz USVs more when subcutaneously administered compared to direct administration into the nucleus accumbens. Error bars represent the S.E.M.
* $p < .05$

As seen in Figure 8 the two routes of amphetamine administration appear to differ in inducing frequency modulation in 50 kHz USVs. To determine if this difference is driven by the same amphetamine-induced modulation of subtype proportions (increasing trill and step calls) found in experiment #1, the 50 kHz call subtypes had to be investigated (for subtype descriptive statistics see Table 5).

Table 5

Average number of calls (raw) of each 50 kHz USV subtype emitted in both intraaccumbens and subcutaneous amphetamine injection conditions.

Condition	Flat	Trill	Step	Ramp	Complex
Intraaccumbens	6	2	2	1	3
Subcutaneous	14	6	5	3	8

As seen in Table 5 subcutaneous amphetamine administration elicited more 50 kHz calls of all subtypes. To determine if the call profile differed between the two routes of administration the proportions of 50 kHz call subtypes had to be investigated. The proportions of trill and step call subtypes were specifically compared across the two injection methods as these subtypes were found in experiment 1 to be influenced by amphetamine. Wilcoxon matched-pairs signed-ranks tests performed on trill ($Z = .56$, $p = .575$) and step ($Z = .14$, $p = .889$) call subtypes across intraaccumbens and subcutaneous amphetamine injections found no difference between these two routes. Moreover, the proportion of flat subtype was not found to be significantly different as well ($Z = .42$, $p = .674$). This finding suggests that the two routes did not differ in the call profiles elicited for either FM or flat 50 kHz USVs (see Figure 9 for graphical representation).

Figure 9. Comparison of Proportions of Subtypes Between Conditions for Experiment #2.

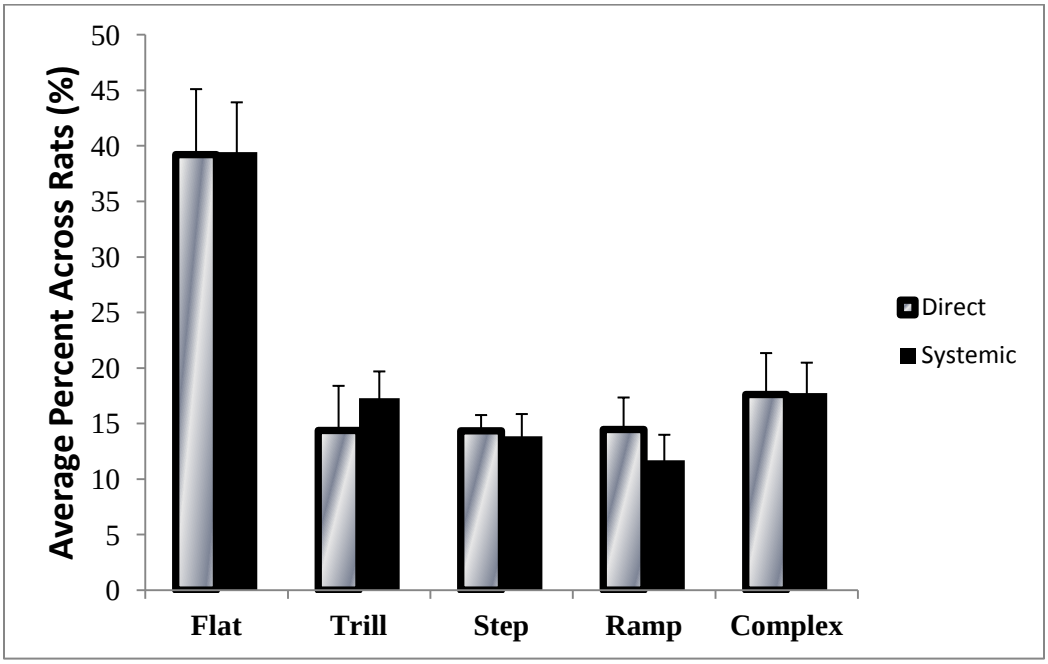

Figure 9. Proportions of 50 kHz USV subtypes compared across intraaccumbens (direct) and subcutaneous (systemic) amphetamine injection conditions for n = 8 rats. This figure illustrates that no significant proportion differences were found for any 50 kHz USV subtype analyzed and there appears to be no difference in the subtype call profile between the two routes of amphetamine administration. Error bars represent the S.E.M. (100% = all calls for one condition).

Experiment #3

As stated in Methods, experiment #3 was comprised of injections designed to determine the role of the nucleus accumbens in the modulation of call profile induced by subcutaneous amphetamine. Injection conditions involved either saline or procaine pre-treatment into the nucleus accumbens followed by subcutaneous amphetamine.

There were several rats excluded from experiment #3 analyses (n = 5). Three of these were excluded again because the injection site was outside of the nucleus accumbens, with a further two removed because of insufficient calls. These exclusions left a useable n of 7 for comparisons between the two conditions of procaine or saline into the nucleus accumbens followed by subcutaneous amphetamine. General acoustic parameters were calculated for each

USV found in the recording session for each rat across both of these conditions; see Table 6 for descriptive statistics.

Table 6

Descriptive statistics for experiment #3 (n = 7) general acoustic parameters of 50 kHz USVs for both Saline + amphetamine and procaine + amphetamine injections.

Condition	Number of Calls Emitted		Peak Frequency (kHz)		Duration (ms)		Bandwidth (Hz)	
	M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
Saline + AMPH	25	12	56.83	5	31.4	6	13631	2557
Proc + AMPH	24	13	58.39	4	30.8	11	13773	3364

As can be seen from Table 6 the two different injection conditions appear to have very similar acoustic parameters across the 50 kHz USVs emitted. The general call parameter of bandwidth was again investigated across the two injection conditions to determine if they significantly differed in frequency modulation regardless of 50 kHz call subtypes. A Wilcoxon matched-pairs signed-ranks test was performed on average bandwidth across the two injection conditions and found no significant difference ($Z = .17$, $p = .866$). This lack of significant difference indicates that the pre-treatment of procaine into the nucleus accumbens did not differ from saline in the amount of frequency modulation induced by subcutaneous amphetamine (see Figure 10).

Figure 10. Average Bandwidth of 50 kHz Calls Comparison for Experiment #3.

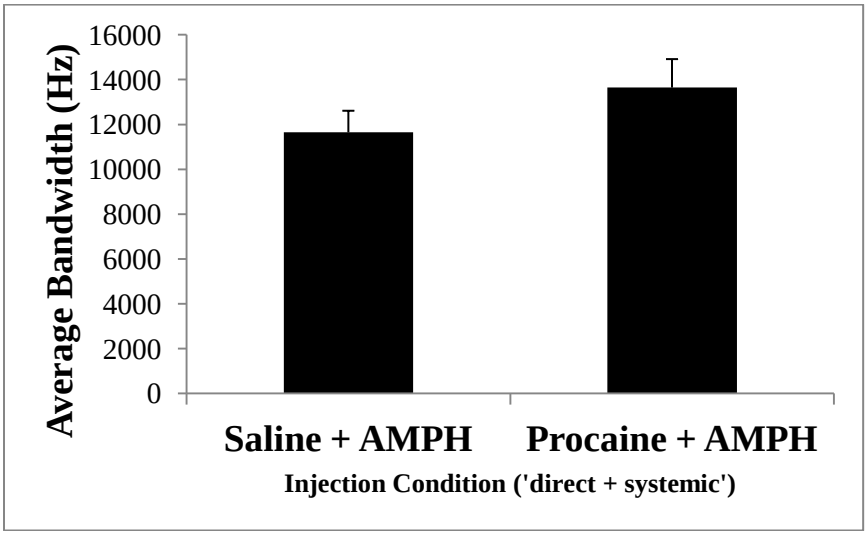

Figure 10. Average bandwidth of 50 kHz USVs after injection of either saline or procaine into the nucleus accumbens followed by subcutaneous amphetamine (AMPH). This figure illustrates that no significant difference was found for the parameter of bandwidth across the two injection conditions indicating they do not differ in the amount of frequency modulation induced by the subcutaneous amphetamine.

To investigate whether the call profile of amphetamine-induced calls differed between the two conditions, the proportions of subtypes had to be taken into account (for general descriptive of raw number of subtypes emitted see Table 7).

Table 7

Average number of calls (raw) of each 50 kHz USV subtype emitted in both saline + amphetamine (AMPH) and procaine (proc) + AMPH injection conditions.

Condition	Flat	Trill	Step	Ramp	Complex
Saline +AMPH	5	7	3	2	5
Proc + AMPH	6	7	4	3	5

As Table 7 shows the raw number of 50 kHz calls emitted for each subtype does not appear to differ greatly between the two conditions. To determine if the proportions of the subtypes in each condition were significantly different Wilcoxon matched-pairs signed-ranks

tests were performed on flat, trill, and step calls similarly to experiments #1 and #2. No significant differences were found for flat (Z = .17, p = .866) as well as trill (Z = 1.19, p = .236) 50 kHz call subtypes. However, the call subtype of step was found to be significantly different between the two injection conditions (Z = 2.37, p = .018). This indicates that the two conditions differed in the average proportion of step calls being emitted with the injection condition involving the procaine pre-treatment of the nucleus accumbens before subcutaneous amphetamine inducing a greater proportion of step calls (see Figure 11).

Figure 11. Comparison of Proportions of Subtypes Between Conditions for Experiment #3.

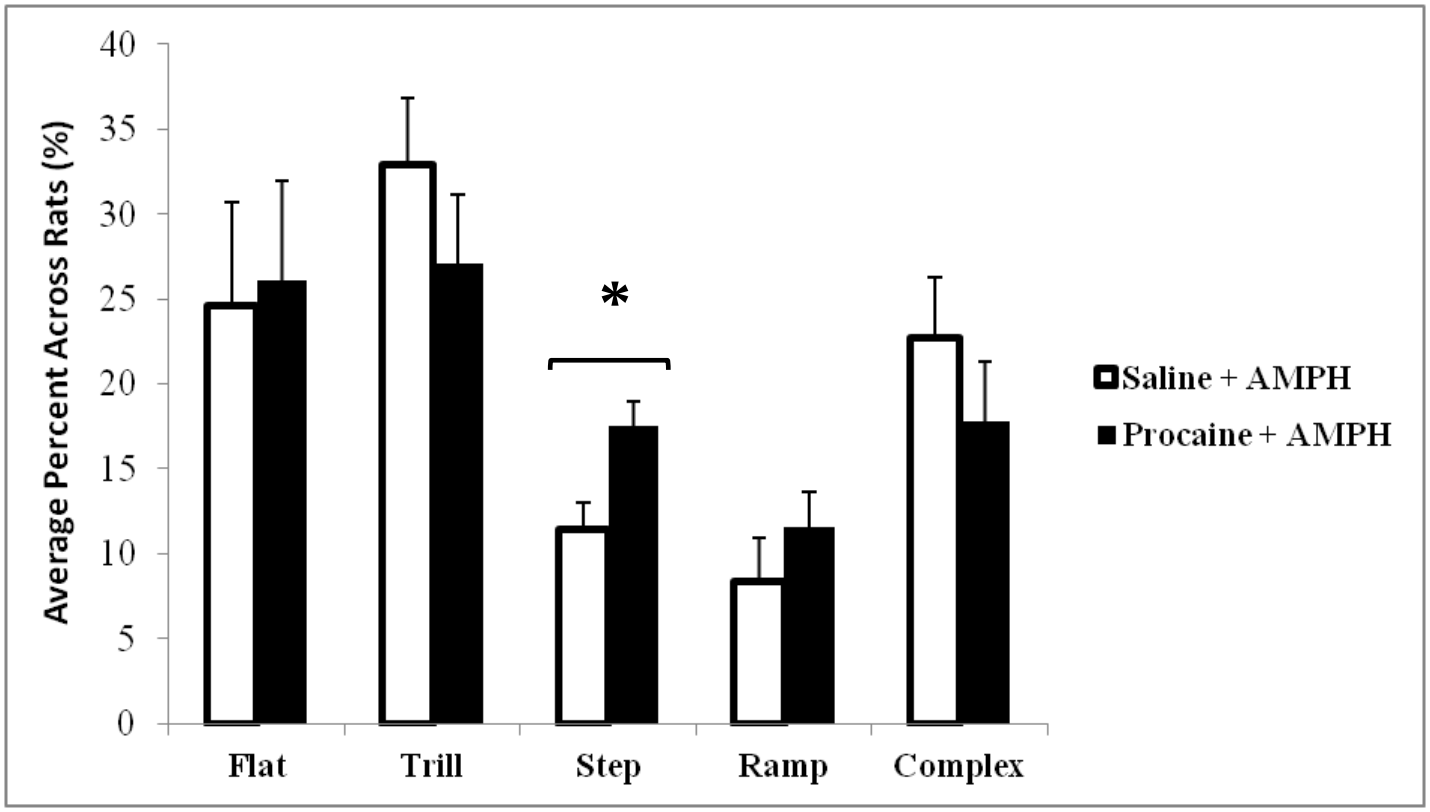

Figure 11. Proportions of 50 kHz USV subtypes compared across saline + amphetamine and procaine + amphetamine injection conditions for n = 7 rats. This figure illustrates that the only 50 kHz USV subtype found to significantly differ in proportion emitted between the conditions was step calls. The pre-treatment of the nucleus accumbens with procaine showed greater proportion of these step calls compared to a vehicle pre-treatment. (100% = all calls for one condition).
* $p < .05$

Control Analyses

As stated in the methods, there were two injections (procaine pre-treatment followed by subcutaneous saline and saline pre-treatment followed by subcutaneous saline), which were designed to provide negative controls for experiment #2 and #3. These injections took place on the same rats involved in experiments #2 and #3 and were carried out after experiment #3. As with these experiments, three rats were excluded due to injection site being outside of nucleus accumbens. Three rats were also excluded for insufficient number of calls (total of n = 6 rats excluded).

General acoustic parameters as well as 50 kHz USV subtypes were collected from these control recording sessions. For general descriptive of the two conditions please see Table 8.

Table 8

Descriptive statistics for control injections' (n = 6) general acoustic parameters of 50 kHz USVs for both Procaine + saline and Saline + saline injections.

Condition	Number of Calls Emitted		Peak Frequency (kHz)		Duration (ms)		Bandwidth (Hz)	
	M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
Proc + Saline	12	6	49.17	9	29.8	8	8803	5764
Saline + Saline	14	7	47.26	8	32.5	11	10134	4579

As can be seen from Table 8 the two control conditions did not appear to deviate greatly in general descriptives. The average number of calls emitted from each rat appeared lower than any other injection condition preceding both control injections. A Wilcoxon matched-pairs signed-ranks test was performed on the general parameter of bandwidth. This was done in order to compare the effect of pre-treatment of procaine into the nucleus accumbens to its saline control when both were followed by subcutaneous saline on general frequency modulation. There was no significant difference found between the two conditions ($Z = .52$, $p = .600$)

indicating that the presence of procaine itself in the nucleus accumbens did not differ from its saline control when followed by subcutaneous saline (see Figure 12).

Figure 12. Average Bandwidth of 50 kHz Calls Comparison for Procaine + Saline Control.

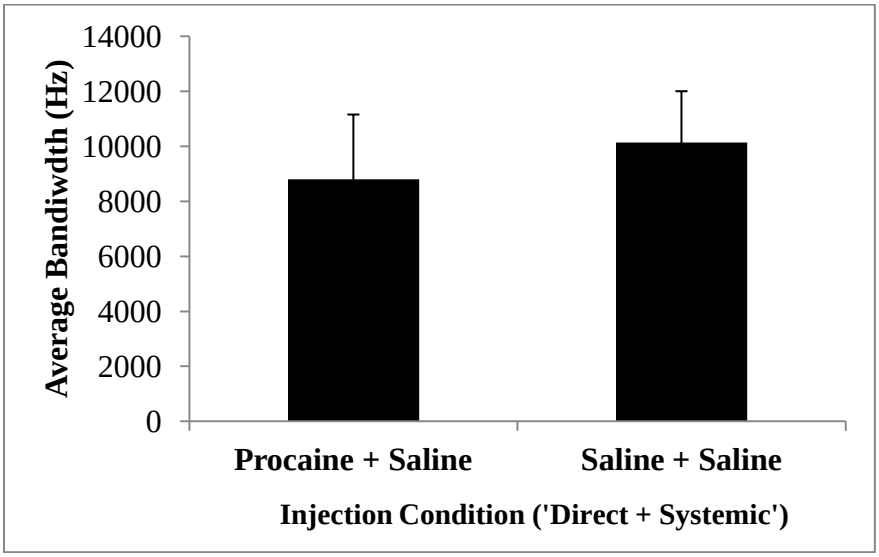

Figure 12. Average bandwidth comparison of 50 kHz USVs in control conditions of procaine or saline pre-treatments followed by subcutaneous saline. This figure illustrates that no significant difference was found across the control conditions for the parameter of bandwidth. This suggests there was no frequency modulation difference across the two injection conditions.

A comparison of procaine + amphetamine and procaine + saline was conducted in order to determine if amphetamine was effectively modulating the call profile of 50 kHz USVs in experiment 3. A Wilcoxon matched-pairs signed-ranks test was performed on the parameter of bandwidth across the two conditions. There was a significant difference found ($Z = 1.99$, $p = .046$) indicating that the procaine pre-treatment followed by amphetamine induced greater average bandwidth than saline. This increased average bandwidth suggests the subcutaneous amphetamine in experiment 3 was increasing frequency modulation in 50 kHz USVs, and the presence of procaine in the nucleus accumbens made no significant difference (see Figure 13).

Figure 13. Average Bandwidth of 50 kHz Calls Comparison for Procaine + Amphetamine Control.

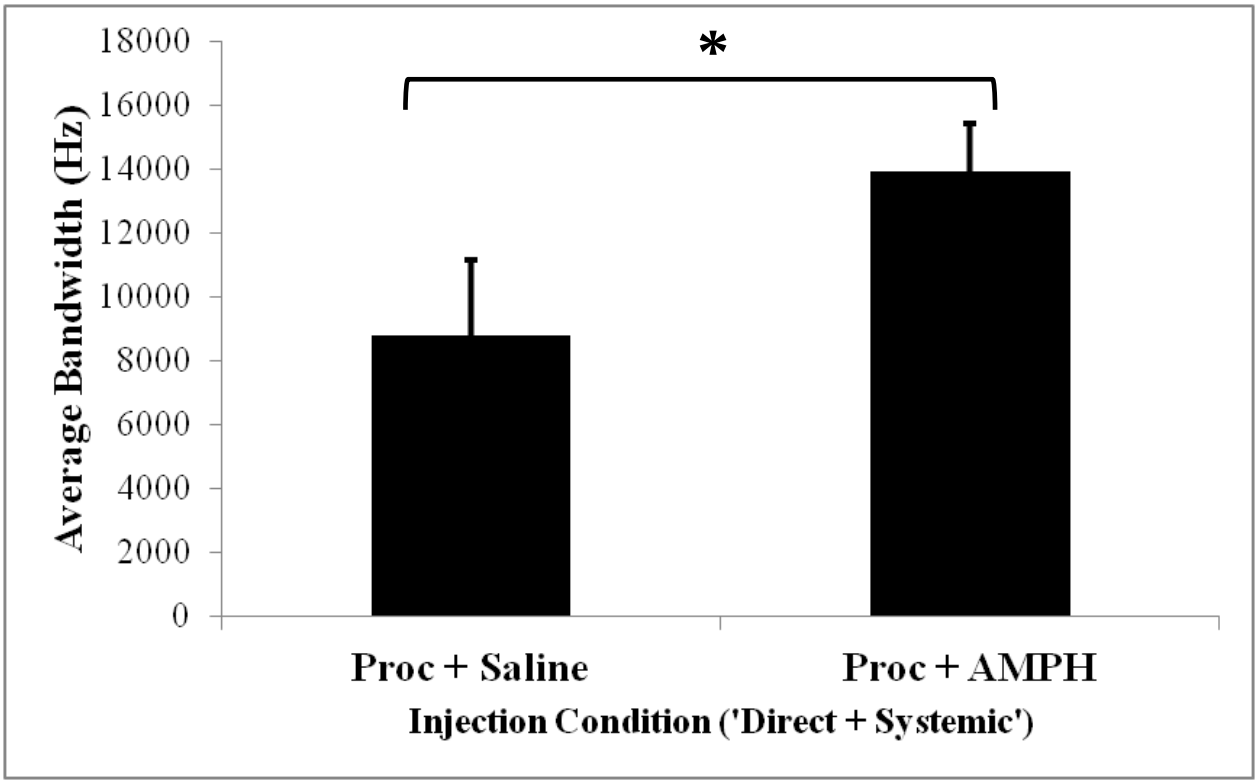

Figure 13. Average bandwidth comparison of 50 kHz USVs between conditions with procaine pre-treatment followed by either subcutaneous saline or amphetamine (AMPH). This figure illustrates that the subcutaneous amphetamine induced significantly greater frequency modulation than saline when the nucleus accumbens was pre-treated with procaine.

Discussion

The purpose of this research was to determine if the nucleus accumbens is a critical structure in the production of 50 kHz USVs that is induced by application of the drug amphetamine. The results found were only partially supportive of the hypotheses. Nevertheless, they raise certain doubts to the established notion that the nucleus accumbens is a critical initiator of 50 kHz USVs.

The results supported hypothesis #1, that subcutaneous amphetamine would modulate the call profile of 50 kHz USVs compared to saline by increasing the proportion of trill and step calls. They only partially supported hypothesis #2, that the two routes (intraaccumbens and subcutaneous) of administration would not differ in their 50 kHz USV call profile. Subcutaneous amphetamine induced a greater amount of frequency modulation, but there was no evidence of differences in proportion of 50 kHz call subtypes. There was no support for the third hypothesis, that the pre-treatment of the nucleus accumbens with procaine would alter the amphetamine-induced 50 kHz call profile. There was no significant difference found in either bandwidth or subtype proportions (except for a procaine condition increase in step calls) and thus procaine did not appear to reduce the expression of trill or step calls.

These three hypotheses were investigated across several experiments, each experiment centred on a given injection procedure. The results of each experiment inform most specifically a corresponding hypothesis and are thus best addressed in sequential order.

Experiment #1 – Hypothesis #1: Amphetamine will induce a shift in call profile such that proportions of trill and step calls will be increased compared to vehicle and amphetamine induced calls will have greater bandwidth compared to vehicle.

The injections involved in experiment one were designed to assess the first hypothesis by injecting amphetamine and saline subcutaneously so that the effect of amphetamine on 50 kHz call profile could be assessed. In line with hypothesis one, the amphetamine condition showed greater average bandwidth of 50 kHz calls when compared to the vehicle condition. Furthermore, as expected from the literature, amphetamine increased the proportion of trill and step calls when compared to saline. This result replicates work done by Wright and colleagues (2010, 2013), and establishes that a subcutaneous application seems to have the same expected effect as intraperitoneal injections. As previously stated, amphetamine is well known to induce 50 kHz calling in rats (Wintink & Brudzynski, 2001; Thompson et al., 2006; Wright et al., 2010). It is also known to be rewarding, as evidenced by high rates of self-administration, and this rewarding nature has been thought to drive the 50 kHz calling (Pickens & Harris, 1968; Thompson et al., 2006). Thus the results of experiment one provide evidence that the induction of positive affect produced by systemic amphetamine may be best indexed by the trill call subtype (which has been used by some researchers as their only FM 50 kHz call; Ahrens et al., 2009). Nevertheless, there is also evidence provided from this experiment that proportions of step calls are also increased by subcutaneous amphetamine, a subtype which has been largely ignored in the literature. Although amphetamine increased the average bandwidth in the 50 kHz calls, it is unclear if this was done across all calls or if it was driven by the increase in large-bandwidth call subtypes (i.e. trill and step) proportions. Regardless, the results from experiment one strongly suggest that amphetamine increases frequency modulation in 50 kHz USVs in the rat.

There is a multitude of possible explanations for the mechanism by which systemic amphetamine may elicit higher proportions of modulated 50 kHz calls. This lack of clarity regarding mechanism is because amphetamine has many different actions beyond its indirect agonism of the dopamine system (Sulzer, Sonders, Poulsen, & Galli, 2005; Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007). It exerts a wide range of effects to increase release of not only dopamine but also norepinephrine and serotonin.

One of the primary ways amphetamine works to increase neurotransmitter release and thereby transmitter activity is by acting on the transporters of monoamines (dopamine, norepinephrine, and serotonin). Although there are a variety of models to explain the mechanism, amphetamine appears to cause an efflux of neurotransmitter in a non-exocytotic fashion either by reversing the transporters or by creating an exchange gradient for diffusion of the transmitters outwards across the uptake carriers (Fleckenstein et al., 2007). In addition, amphetamine also influences the vesicular transporters for monoamines. This influence on vesicular transport likely results from amphetamine's weak base properties that disrupt the electrochemical gradient necessary for transmitter substance to be transported into vesicles (Feldman, Meyer, & Quenzer, 1997; Fleckenstein et al., 2007). This disruption has the effect of increasing cytoplasmic stores of transmitter substance at the expense of the vesicular pool by inhibiting the vesicular uptake process and also by evoking release from vesicular storage sites (Feldman et al., 1997). Thus this mode of action likely works in conjunction with the exchange diffusion/reverse transport mechanism; increased cytoplasmic store of transmitter substance likely then increases the possible efflux across the transporter (Fleckenstein et al., 2007).

Though amphetamine works in a variety of ways on multiple neurotransmitters its actions on the dopamine system are of particular interest for the effects found in experiment #1. This is

because the mesolimbic dopamine system is widely recognized to underlie the addictive and rewarding properties of amphetamine (Sulzer et al., 2005). In addition, more than other affected monoamines dopamine degradation from the cleft is almost entirely reliant on reuptake mechanisms (Feldman et al., 1997). This indicates that it is likely the most affected neurotransmitter by amphetamine because of its clearance from extracellular fluid requiring working reuptake mechanisms via the dopamine transporter (Sulzer et al., 2005). There is also good evidence that the effect of amphetamine is to dramatically increase dopamine specifically in the nucleus accumbens by activation of the mesolimbic system terminals (Moghaddam & Bunney, 1989). If amphetamine increases the proportion of trill and step calls by activation of the mesolimbic dopamine system then it may be the case that these 50 kHz subtypes index positive affect. This mesolimbic dopamine mechanism would converge with evidence that 50 kHz calls can be elicited by other dopamine agonists with very different modes of action (Brudzynski et al., 2012; Wright et al., 2013), and that 50 kHz calls are highly associated with positive affect (Knutson et al., 1998; Knutson et al., 1999; Burgdorf et al., 2000).

In contrast, it is possible that the actions of amphetamine to increase norepinephrine may also account for the results found in experiment #1. Amphetamine is well known to increase general arousal and thus trill and step 50 kHz subtypes may actually index level of general arousal. This general arousal explanation would fall in line with recent research put forward by Wright and colleagues (2012) which argues that alpha- and beta-adrenergic receptor activation is necessary for the amphetamine modulation of 50 kHz call profile. This explanation alone, however, would fail to account for the association with positive affect that 50 kHz calls have generally and it is questionable whether arousal can be meaningfully parsed apart from affective states. Thus, there may be a catecholamine mechanism, involving both dopamine and

norepinephrine, driving the amphetamine-induced modulation of 50 kHz call profile. The greater explanatory power of the role of dopamine and the mesolimbic dopamine system, however, leads to the conclusion that this contribution may be the most critical, as evidenced by dopamine depletion studies (Burgdorf et al., 2007; Ciucci et al., 2009).

There are a number of limitations with experiment #1. The subcutaneous injections of amphetamine were done at a single dose; this dose was utilized simply to induce calls to compare. Usage of multiple doses (enabling creation of a dose-response curve) may have further elucidated some of the mechanism by which amphetamine is inducing the shift in 50 kHz call profile. Furthermore, the vehicle condition which served as a control may not have been as effective as an alternative injection condition which pharmacologically induced 50 kHz calls but through a different mechanism (e.g. apomorphine). This alternative control would have allowed more firm conclusions to be drawn about how specific the results of experiment #1 are to amphetamine effects and not to general pharmacological effects. Nevertheless, evidence from similar work which involved a dopamine reuptake inhibitor (GBR 12909) found that the effect was unique to amphetamine and was not shared with this alternative pharmacological agent (Wright et al., 2013).

Evidence from experiment #1 indicates that subcutaneous amphetamine induces greater frequency modulation of 50 kHz calls, showing also increased proportions of trill and step call subtypes. Thus, the question of whether this effect of amphetamine was taking place through its actions on the well established structure of nucleus accumbens or by activating the whole mesolimbic dopamine system generally was raised and experiment two was designed to investigate this question.

Experiment 2 – Hypothesis 2: Direct application of amphetamine into the accumbens will induce 50 kHz calling, with proportions of call subtypes and bandwidth not differing from those induced by systemic application of amphetamine.

The injections in experiment #2 were designed to test the second hypothesis by comparing two routes of application (direct intraaccumbens and systemic subcutaneous) of amphetamine. The results were partially supportive of hypothesis #2. As expected, the two routes of application did not seem to differ in the proportions of 50 kHz call subtypes elicited by amphetamine. Contrary to the hypothesis, they did differ significantly in bandwidth (and number of calls), indicating that subcutaneous amphetamine induced a greater amount of frequency modulation. This bandwidth difference was not driven by differences in subtype proportions and thus must be reflected in the acoustic parameters of the calls themselves. Because the two conditions did not differ in proportions of call subtypes, it suggests that across all calls or within specific calls (e.g. trill calls) there was a greater amount of frequency modulation in the systemic condition.

These results may seem counter intuitive, given that amphetamine has been so well demonstrated to induce calls when administered directly into the nucleus accumbens (Burgdorf et al., 2001; Thompson et al., 2006). Moreover, the nucleus accumbens is central to the reward association of the mesolimbic dopamine system (Ikemoto & Panksepp, 1999; Alcaro et al., 2007). A great amount of the dopaminergic innervations in the mesolimbic system are found in the ventromedial portion of the striatum (Ikemoto, 2007) and extracellular dopamine levels in the nucleus accumbens are sensitive to VTA activation (Westerink et al., 1996). Thus increasing dopamine activity in the nucleus accumbens shell is certainly capable of inducing the high rates of 50 kHz calling that are associated with positive affect (Knutson et al., 2002; Brudzynski et al.,

2012). This accumbens activation, however, is not necessarily the only avenue, as the midbrain dopamine neurons have extensive projections to many structures beyond the nucleus accumbens (Swanson, 1982; Oades & Halliday, 1987). These structures (ventral pallidum, olfactory tubercle, lateral septum, etc.,) that receive extensive dopaminergic innervations from the VTA make up what is broadly conceptualized by some as the 'general-purpose reward-seeking' system (Alcaro et al., 2007; Panksepp, 2011). Moreover, there are also structures (midbrain raphe nuclei, rostromedial tegmental nuclei, etc.,) outside of the mesolimbic dopamine system that may be active in mediating reward or interacting with this 'seeking' system through non-dopaminergic mechanisms (Ikemoto, 2010). Though there is extensive evidence that these structures are associated with reward/positive affect, there is a lack of research into their association with 50 kHz USVs. Nevertheless, there is preliminary data that injection of a dopamine agonist into the lateral septum can elicit 50 kHz USVs (Silkstone & Brudzynski, unpublished results). Thus, when consideration is given to the broader systems and structures in the mammalian brain associated with positive affect, as activated by systemic amphetamine, it is not counter intuitive that systemic amphetamine elicited greater frequency modulation than intraaccumbens amphetamine.

The results of experiment #2 showed systemic administration of amphetamine produced greater bandwidth than local application, thus it seems that this route likely activated the generation circuit for FM 50 kHz calls more strongly than intraaccumbens injections. There is evidence showing the overlap of this generation circuit with the mesolimbic dopamine system (Burgdorf et al., 2007) and, as stated previously this dopamine system involves more than the nucleus accumbens shell. Thus, the systemic amphetamine was free to act on any structure in the mesolimbic dopamine system and beyond.

Intracerebral microdialysis in the rat brain has shown that amphetamine-induced dopamine release is particularly great in the nucleus accumbens (Sharp, Zetterstrom, Ljungberg, & Ungerstedt, 1987). However, there is also evidence that other structures (for instance the medial prefrontal cortex) are also particularly sensitive to amphetamine (Moghaddam & Bunney, 1989). It is therefore reasonable to conclude that amphetamine may have increased dopamine activity beyond the nucleus accumbens. The variety of possible amphetamine mechanisms mean that the systemic administration may have acted on other monoamine systems. For instance, amphetamine may have increased bandwidth by increasing arousal through its effects on norepinephrine. This effect of general arousal may have created greater frequency modulation (potentially as a by product of locomotor activity) though this would not be supported for subtype proportions.

The results of experiment #2 also showed no significant difference in 50 kHz subtype proportions elicited in both conditions. This lack of significant difference suggests that the administration of amphetamine into the small localized area of the ventromedial portion of the nucleus accumbens shell was sufficient to induce calls in a comparable fashion proportion-wise to systemic activation of the mesolimbic dopamine system. It is possible to interpret this result as evidence against the idea mentioned previously that trill and step calls index general arousal. This interpretation is supported because there was no proportion differences in the 50 kHz subtypes emitted between conditions whereas general frequency modulation did appear greater in the subcutaneous condition than the direct. Thus the call subtype profile is potentially independent from the arousing properties of amphetamine (which appear to require system wide activation). Furthermore, it indicates that injection of amphetamine into the nucleus accumbens

shell should be sufficient to produce the modulatory effects on 50 kHz call profile previously established with amphetamine.

There were several limitations associated with experiment #2 that put constraints on the interpretations and conclusions derivable from the results. The injection conditions were embedded in a larger injection set and thus could only be counter balanced between each other and not immediately with any vehicle control. Moreover, there is difficulty in determining the possible effect of amphetamine dose between the two conditions as a direct comparison of dosage is impossible. One step towards determining the effect of dose between the two routes of administration is to compare dose-response curves of the respective routes. Even this, however, would be difficult to interpret and may not provide meaningful answers. In experiment #2, a dose of amphetamine that would sufficiently elicit 50 kHz calls for each respective route of administration was deemed satisfactory for the initial exploration of the effect of route on calling. A further limitation with experiment two was that because of the relatively low amount of calls in both conditions it was not possible to take into account the time course of 50 kHz calling. These time course results may have yielded insights into the effect of different pharmacokinetics on 50 kHz subtype production.

The results from experiment #2 indicate that subcutaneous amphetamine activates the generation circuit for 50 kHz calls more strongly than a local application into the nucleus accumbens shell. This interpretation was evidenced by the finding of greater frequency modulation of 50 kHz calls induced from systemic amphetamine compared to intraaccumbens. Moreover, there was no difference found in proportions of subtypes emitted in both injection conditions. This finding indicates that the nucleus accumbens has a sufficient role in initiating the amphetamine-induced modulation of 50 kHz call profile. To determine if this structure has a

necessary role experiment #3 was designed to investigate if a blockade of the nucleus accumbens would alter the amphetamine modulation.

Experiment #3 – Hypothesis #3: Amphetamine-induced calls with procaine blockade of the nucleus accumbens will have reduced proportion of trill and step calls as well as reduced bandwidth compared to amphetamine-induced calls without procaine blockade.

The injections in experiment #3 were designed to test hypothesis #3 by pre-treatment of the nucleus accumbens shell with either procaine or saline followed by subcutaneous amphetamine. It was hoped that the role of the nucleus accumbens could thus be assessed in the amphetamine-induced modulation of 50 kHz call profile. The results failed to support hypothesis #3. There was no significant reduction in trill and step proportions of 50 kHz calls (which instead were actually significantly increased for the case of step calls) in the procaine pre-treatment condition, nor was there any significant difference in bandwidth between the two conditions. These results indicate that the presence of procaine in the nucleus accumbens shell had no significant reductive effect on the amphetamine modulation of 50 kHz call profile. This finding may suggest that the nucleus accumbens shell is not involved in the modulation of 50 kHz subtype proportions, even with dopamine activity in the shell likely intact. These results are contrary to what the results from experiment #2 indicated -- that dopamine activity in the nucleus accumbens shell was sufficient for the amphetamine induction of 50 kHz call profile.

These results also seem contrary to what was expected based on the well-established literature showing that the nucleus accumbens is highly associated with 50 kHz calling (Thompson et al., 2006,) and integral to the function of the mesolimbic dopamine system (Westerink et al., 1996). Research which has used 6-hydroxydopamine (6-OHDA) and electrolytic lesions to disrupt this mesolimbic system has shown significant reductions in 50 kHz

calling (Burgdorf et al., 2007). This research however did not disrupt the mesolimbic system specifically downstream from the nucleus accumbens shell, thus it is possible that the results from experiment #3 are in line with this literature.

The explanation for the observed results in experiment #3 is not obvious. The dopamine mechanism by which amphetamine may modulate the call profile (discussed under experiment one heading in this section) is not affected by the application of procaine. Procaine is a local anaesthetic and reversibly blocks the activity of sodium channels from the intracellular side of the membrane (Butterworth & Strichartz, 1990; Creveling et al., 1990). It also has similar properties to cocaine in that it blocks dopamine reuptake, though it is more selective for blocking sodium channels and a less potent reuptake blocker (Hernandez, Guzman, & Hoebel, 1991; Woodward et al., 1995). This effect of procaine was intended to allow for the action of amphetamine to effectively induce 50 kHz calling (it is even possible that procaine could amplify the activity of amphetamine generally as it has some property of reinforcement; Wilcox, Paul, Woolverton, 1999). Thus the activity of procaine in the nucleus accumbens shell should not have prevented amphetamine from increasing dopamine release (any competitive binding for the dopamine transporters would still have led to increased extracellular dopamine). It should have, however, prevented action potential propagation and conduction from the shell to downstream structures.

The results finding no difference in either bandwidth or subtype proportions suggest that the accumbens shell and its downstream targets have no role in the amphetamine modulation of 50 kHz calls. This finding appears to directly contradict the results of experiment #2, which indicated the accumbens shell did indeed play a sufficient role in the amphetamine modulation of call profile (though not bandwidth). The findings from Ikemoto and Witkin (2003) suggest that

the neurons in the ventromedial nucleus accumbens may have less sensitivity to the anesthetic properties of cocaine and procaine than those in the core region. It is possible that this may account for the results of experiment #3 in that the sub-regions of the nucleus accumbens differ greatly in their susceptibility to being anesthetised. Furthermore, the nucleus accumbens is a complex structure with a great array of cell types and inputs (Britt et al., 2012). It is conceivable that because the inducing amphetamine was given systemically it activated a great number of limbic structures that had inputs into non-anesthetised areas of the nucleus accumbens. The injection area in which the drug could diffuse and have effect on sodium channels was relatively miniscule compared to the total size of the accumbens shell (which due to its anatomical shape is impossible to pharmacologically saturate without major destruction). In addition, because procaine blocks activated sodium channels it requires depolarization of the membrane to have functional effect. Dopamine in the accumbens may have a largely inhibitory role; there is evidence that dopamine receptor (D2) activation depresses cell firing (Yim & Mogenson, 1986; Nikola et al., 2000). Thus, procaine may have had reduced effectiveness as an anesthetic in the nucleus accumbens shell. Nevertheless, there is extensive evidence that the actions of dopamine are complex and should not be classified as excitatory or inhibitory (Spanagel & Weiss, 1999; Nikola et al., 2000). Regardless, there are a large number of GABAergic cells in the nucleus accumbens shell (Nikola et al., 2000) and procaine has been shown to actually enhance GABA receptor function if in low concentration (Hara & Sata, 2007). Therefore, it is unclear whether the procaine mechanism was effective in reducing conduction in the nucleus accumbens for a variety of reasons.

There are a number of limitations in experiment #3 that may help contextualise the lack of results and represent possible areas for future improvements in similar studies. Because the

administration of amphetamine was only subcutaneous the procaine pre-treatment had no non-vehicle control (which could have been achieved by a counter balanced amphetamine injection into a procaine pre-treated accumbens). This type of control would have elucidated some of the questions raised above pertaining to the extensive limbic inputs into the ventral striatum which may have been activated by systemic amphetamine. Moreover, the dosage of procaine may not have been sufficient. Some studies have used over four times as much as used in the current study (Morency & Beninger, 1986). Given the anatomy of the target area and the size of the structure, a greater dose in a greater volume of vehicle may have produced more notable results. Beyond all of this lies the question of whether the procaine was actually efficacious as an anesthetic. The control injections were designed to account for this possibility.

Controls

The control injections and comparisons were made so that the effectiveness of the procaine blockade of the nucleus accumbens shell could be assessed without amphetamine induction of 50 kHz calling.

The comparison between procaine and saline pre-treatment of the nucleus accumbens followed by saline found no significant difference in bandwidth between the two conditions. This lack of significant difference does not support the assumption that the procaine was effectively blocking conduction in the accumbens shell. Moreover, the comparison between procaine + amphetamine and procaine + saline, where in both conditions the nucleus accumbens was pre-treated with procaine, revealed a significant difference in bandwidth. The condition with amphetamine induction of calls showed a greater average bandwidth compared with saline. This finding suggests that the presence of procaine did not prevent amphetamine from increasing frequency modulation.

It is possible that procaine was still efficacious as an anesthetic and that these results indicate that frequency modulation of calls is relatively independent of the accumbens shell. There is little reason, however, for confidence in this interpretation. There were unfortunately a low number of total calls in both procaine-saline control conditions (due to being the last injection comparison in a broader injection set). This low number prevents meaningful analyses to be done on the number of calls made in the conditions to attempt to determine if the presence of procaine had any general reductive effect. Nevertheless, the amount of calls made (though relatively low), appear descriptively equal in the two groups and thus do not support the effectiveness of the procaine. The procaine-amphetamine control conditions suggest that amphetamine increased the number of 50 kHz calls, however this injection took place earlier in the set and thus it not be possible to have an accurate comparison in this regard.

General Discussion

The present study is the first to demonstrate that subcutaneous injections of amphetamine and it confirms the established amphetamine-induced modulation of 50 kHz call profile by increasing proportions of trill and step subtypes. It is also the first to indicate that subcutaneous and intraaccumbens routes of amphetamine administration do not appear to differ in their 50 kHz call profile, though systemic may activate the generation circuit more strongly. The role of the nucleus accumbens shell and its downstream targets in this amphetamine-induced modulation still remains unclear however, as the results of procaine pre-treatment are ambiguous and difficult to meaningfully interpret.

The findings of this study serve most directly to illustrate the possible utility in investigating the frequency modulation element in 50 kHz USVs. The variety of 50 kHz subtypes raise many questions related to how frequency modulation may be important in the

communication between conspecifics which are not answered by this current research. However, the findings that amphetamine induces an increase in specific proportions of subtypes whether given systemically or directly into the accumbens shell serves as an important step towards answering these questions. The notion supported by these findings that the nucleus accumbens shell is involved in this call profile modulation provides first evidence that there is a neuro-anatomical basis for some call subtype categorization. Moreover, the evidence that subcutaneous administration of amphetamine elicits greater frequency modulation compared to local application in the shell may suggest the function of other structures beyond the accumbens are implicated in generating FM 50 kHz calls. This evidence establishes a basis for future investigations into which structures these may be (lateral septum, olfactory tubercle, ventral pallidum etc.,).

There were a number of broad limitations with this research beyond those limitations already mentioned for each specific experiment. Because of the nature of the injection set which utilized the same rats across experiment #2, #3, and control injections there was limited freedom to counter balance. Moreover, the experiments required sequential ordering as physiological disruption occurs with each intracerebral pharmacological application. This trait of intracerebral applications created a problem in interpreting the latter injections (controls) due to the lower number of calls that may have been caused in part by this sequential ordering. The low number of rats that were able to be used in any given experiment severely limited the comparisons possible and thus the conclusions that could be drawn from the data. A higher number both of rats and of calls across all conditions may have allowed greater resolution in the 50 kHz subtypes investigated (broader than 5 major types; potentially 10 different types). Throughout the current study the mechanism by which amphetamine induced the 50 kHz call profile modulation was

unknown. There may have been some elucidation of this mechanism if alternative dopaminergic agonists had been used across the experiments as pharmacological controls. Unfortunately, a number of dopaminergic drugs were tried but could not successfully induce calling across both subcutaneous and intracerebral conditions and thus had to be excluded from use in this study.

Future investigations must more specifically address the nature of the 50 kHz subtypes with behavioural research on the effect of various subtypes on conspecific behaviour. These investigations may provide a much greater ethological basis for subtype categorization than is currently available. Moreover, investigations into the receptor subtypes implicated in the generation of various 50 kHz subtypes will also provide a neurophysiological basis for categorization. Future research into the mechanism of amphetamine on influencing proportions of 50 kHz subtypes may utilize varying dosages to determine if the mechanism of amphetamine to modulate is dose-dependent. Moreover, investigations into the role of the accumbens shell may compare a replication of a procaine blockade with more conventional lesions such as electrolytic or injections of GABA agonists/dopamine antagonists.

In conclusion, a great amount of work is still required in order to understand the function and role of 50 kHz FM subtypes. Nevertheless, there is reason to believe that the trill and step subtypes may best index positive affect. Whether these FM subtypes are increased in other affect-evoking contexts or are merely a pharmacological by product requires further research on the nature of these calls. Furthermore, this research suggests a system broader than the pathway from VTA to the nucleus accumbens shell exists as part of the generation circuit responsible for FM 50 kHz USVs.

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